CD8+ T cell attrition in chronic hepatitis B virus infection

A thesis presented by A.Ross Lopes to the University of London
for the degree of Doctor of Philosophy

August 2007

The Windeyer Institute
Department of Immunology and Molecular Pathology
Division of Infection and Immunity
University College London
United Kingdom

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

Declaration

All the work presented in this thesis is the authors own, other than where clearly stated.

A.Ross Lopes

Abstract

Hepatitis B virus (HBV)-specific CD8+ T cells are critical for a successful immune response to HBV infection. These cells are markedly diminished in patients that are unable to contain the virus and the mechanisms contributing to their depletion are not well-understood. The aim of this work was to further dissect this response in individuals with chronic infection.

Studies of the HBV-specific CD8+ T cell immunodominance hierarchy demonstrated that persistent infection was associated with a hyporesponsiveness that was exacerbated with increasing antigenic load. The immunodominant core 18-27-specific response appeared to be particularly prone to deletion whereas a population of envelope 183-191-specific CD8+ T cells managed to persist despite excessively high viral loads but with an inability to exert effective antiviral function.

cDNA microarrays were used to compare these responses in resolved and chronic infection and helped identify a group of functionally-related genes that were selectively up-regulated in HBV-peptide stimulated CD8+ T cells from individuals with chronic infection. The most striking of these was the pro-apoptotic mediator of cross-tolerance induction, Bim, whose expression was confirmed to be upregulated at the protein level. Inhibition of Bim-mediated apoptosis resulted in enhanced detection of HBV-specific CD8+ T cells both in culture and directly *ex vivo*. In accordance to Bim-mediated deletion of $CD127^{low}$ CD8+ T cells, the HBV-specific CD8+ T cells surviving in chronic HBV infection were found to be $CD127^{high}$. These populations had elevated levels of the anti-apoptotic protein Mcl-1, suggesting they had been subject to IL-7 mediated rescue from apoptosis.

This study indicates that Bim-mediated attrition of HBV-specific CD8+ T cells contributes to the inability of these populations to persist and control viral replication.

Acknowledgments

First and foremost, I thank Mala Maini both for giving me the opportunity to pursue a doctorate under her tutelage and for the first-class scientific guidance, continuous encouragement and inexhaustible support that she provided throughout. She far surpassed the standard duty of a supervisor and for that I am truly grateful.

Several additional people were involved at various stages of the study. In particular, I thank Antonio Bertoletti, Paul Kellam, Charles Bangham, Adrian Heaps, Andrew Worth, and Benjamin Chain for valuable feedback, advice and technical expertise. I also thank A.J. Wolfman McQuaid who despite the unrelenting critique offered rejuvenating companionship.

Common themes that occurred during the course of this challenging degree included frustration, anger, and at times despair. It is not that I coped well with those feelings, but instead, my wife did. Caro mia, I celebrate this great achievement because of the extraordinary enduring patience, positivity and confidence that you shared with me in return.

And finally, through late nights by a window overlooking Kentish Town Road and solitary weekends in an office beside the BT tower, the focus and urgency to complete I drew from the excited expectation of the arrival of my baby, Maura.

Table of contents

List of figures

List of tables

List of common abbreviations

ALT	Amino-L-transferase
APC	Antigen presenting cell
aRNA	anti-ribonucleic acid (RNA complementary sequence)
Bim	Bcl2 interacting mediator
CD	Cluster of differentiation
cDNA	complementary deoxyribonucleic acid
CDR	Complementarity determining region
CH	Chronic hepatitis
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell
DNA	Deoxyribonucleic acid
dsRNA	Double stranded RNA
EBV	Epstein bar virus
ER	Endoplasmic reticulum
FACS	Fluorescence activated cell sorter
FDR	false discovery rate
HBcAg	Hepatitis B virus core antigen
HBeAg	Hepatitis B virus precore-core antigen
HBpAg	Hepatitis B virus polymerase antigen
HBsAg	Hepatitis B virus surface/envelope antigen
HBxAg	Hepatitis B virus X antigen
HBV	Hepatitis B virus
HCMV	Human cytomegalovirus
HDA	Heteroduplex Analysis
HIV	Human Immunodeficiency Virus
HLA	Human leukocyte antigen
HuPo	Human acidic ribosomal protein
IFN-α	Interferon alpha
IFN-β	Interferon beta
IFN-γ	Interferon gamma

IL-(*n*)	Interleukin – (designated number)
LHB	large HBsAg
LSEC	Liver sinusoidal epithelial cell
MHB	medium HBsAg
MHCI	major histocompatability complex, class I
MHCII	major histocompatability complex, class II
mRNA	messenger ribonucleic acid
NF-Kβ	Neural factor Kappa Beta
NK	Natural killer
ORF	open reading frame
PAMP	pathogen-associated molecular pattern
pAPC	Professional antigen presenting cell
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
FCS	Foetal calf serum
PCR	Polymerase chain reaction
PRR	Pattern recognition receptor
RNA	Ribonucleic acid
SAM	Significance analysis of mciroarrays
SHB	small HBsAg
SOM	Self organising maps
SNR	Signal to noise ratio
ssRNA	single stranded RNA
Tc	T cytotoxic
TCR	T cell receptor
TGF-β	Transforming growth factor beta
Th	T helper
TNF-α	Tumour necrosis factor alpha
TNFR	Tumour necrosis factor receptor

Overview of the thesis layout

The main introduction begins with a brief description of persistent viral infection, followed by a focus on host immune evasion strategies, and continues with a detailed description of the pathogen focused on in this study; the hepatitis B virus. This is followed by a description of the main compartments of the overall immune response with a particular focus on antiviral immunity towards HBV. The chapter concludes with a detailed description of microarray gene expression analysis.

The material and methods contain details of all the protocols and reagents (except standard laboratory consumables) that were used to execute this study.

Chapter three is a study of the immunodominance hierarchies associated with resolved and chronic HBV infection that also served to identify the antiviral responses that were further examined in chapters four and five.

Chapter four covers the initial testing, validation and subsequent use of cDNA microarrays for the study of HBV-specific CD8+ T cells derived from individuals with resolved and persistent HBV infection.

Chapter five is a detailed investigation of the involvement of one candidate gene (identified in chapter 4) in HBV-specific CD8+ T cell attrition in individuals with persistent HBV infection.

Future directions are also provided indicating potential studies that can be pursued to build upon the work in this thesis.

During the course of this investigation, work in addition to the studies in HBV infected individuals, led to the publication of a paper comparing the CD8+ T cell response associated with HIV-1 and HIV-2 infection; a copy is provided at the end of the thesis.

1 Chapter 1 – Introduction

Humans are continuously exposed to viral microbes that attempt to invade and colonise the host. Primary resistance is based on physical barriers but when these are breached the pathogen encounters a secondary highly organised system of defence. This immunity is exerted through multiple specialized cell types that differ principally in the way that they recognise and respond to the pathogen. Among these subtypes, CD8+ T cells have the unique capability of directly recognizing virally infected cells and subsequently exerting appropriate antiviral functions to contain the pathogen. Several studies have demonstrated that chronic infection is associated with defects in both the quantity and quality of this CD8+ T cell response. Understanding these defects is critical to the development of immunotherapeutic strategies aimed at overcoming persistent viral infections.

1.1 Viral pathogens

Viruses exhibit tremendous diversity but can be broadly categorized according to their genetic make-up. According to the Baltimore classification system that groups viruses into families according to their genome type and method of replication, the three main groups consist of DNA viruses (including double and single stranded DNA viruses), RNA viruses (including positive-sense single stranded RNA viruses, negative-sense single stranded RNA viruses and double stranded RNA viruses) and reverse transcribing viruses (double-stranded reverse-transcribing DNA viruses and single-stranded reverse-transcribing RNA viruses including retroviruses). Viral persistence refers to perseverance of these pathogens in either the host or the environment. This thesis focuses on the former: virus-host interactions.

Viral microbes utilize either acute or persistent patterns of infection and these are reflective of two different survival strategies associated with distinct evolutionary ecologies (Villarreal et al., 2000).

1.2 Acute and persistent viral strategies

The acute viral life strategy involves a transient form of host infection that is limited by the host immune system. Due to the limited window of opportunity, the pathogens must be able to rapidly transmit to a new host to ensure the preservation of the species. Several human viruses that cause epidemic disease (influenza, measles, polio, smallpox) use this strategy. They are usually associated with high mutation rates, diverse quasi-species, and the ability to replicate in multiple host species, particularly those that exhibit congregational populations (Villarreal et al., 2000). The majority of DNA viruses (herpesvirus and papilloma viruses) utilise a persistent life strategy that is highly species-specific (Bernard, 1994; Umene and Sakaoka, 1999). These viruses are not as dependent on the host population structure; natural transmission occurs either vertically from parent to offspring or horizontally by sexual contact or bites.

1.3 Viral evasion of the host immune system

Successful viral persistence relies on the ability of the pathogen to effectively counter the immune response. Several mechanisms, that that target both innate and adaptive immunity, have been extensively reviewed (Barber, 2001; Farrell and Davis-Poynter, 1998; Finlay and McFadden, 2006; Hilleman, 2004; Iannello et al., 2006; Klenerman and Hill, 2005; Lieberman et al., 2002; Oldstone, 2006; Peterhans et al., 1999; Villarreal et al., 2000; Vossen et al., 2002); these can be grouped according to the survival strategy they employ (Xu et al., 2001).

1.3.1 Escape

Effective viral control relies on the ability of antiviral CD8+ T cells to recognize pathogen-derived peptides that are displayed on MHC class I complexes (MHCI) on the surface of target cells; some viruses have evolved mechanisms that interfere with this process. For example, herpes simplex virus utilises infected cell protein 47

(ICP47) to inhibit peptide transport from the cytosol to the endoplasmic reticulum (ER) (Ahn et al., 1996b; York et al., 1994). Human cytomegalovirus uses the US6 gene product to block TAP-mediated peptide translocation (Ahn et al., 1997; Lehner et al., 1997), US2 and US11 gene products to dislocate the MHCI heavy chain, and the US3 gene product to retain MHCI within the ER (Tortorella et al., 2000).

Alternatively, HCMV and HIV-1 have evolved ways to direct the MHCI complex to endosomal compartments for degradation (Le Gall et al., 1998; Wiertz et al., 1996a). Additionally, in order to avoid susceptibility to natural killer (NK) cell–mediated killing, viruses such as HIV-1 selectively downregulate MHCI complexes critical to antiviral function (HLA-A and HLA-B) but maintain expression of the immune inhibitory receptors (HLA-C and HLA-E) (Cohen et al., 1999).

Instead of interfering with the presenting complex, certain pathogens, particularly RNA viruses that utilize error prone reverse transcriptases, escape detection by mutating critical antigenic determinants (Bertoletti et al., 1994; Klenerman et al., 1994; Koup, 1994).

Lastly, viral latency, an effective form of viral escape, enables pathogens such as EBV, HSV-1 and HIV-1, to remain invisible by minimising viral protein expression. These viruses become active when the cell is activated, during which time they replicate, infect other cells and transit to other hosts (Khanna et al., 2004; Tsurumi et al., 2005).

1.3.2 Resistance

In order to survive, viruses must ensure that the cells they infect do not undergo programmed cell death before they can generate a sufficient quantity of infectious progeny. Cellular apoptosis can be mediated through several pathways; these involve multiple death mediators that present a variety of targets for viral interference. KSHV disrupts recruitment of caspase 8 to the Fas-associated death domain (FADD) via a

product of the ORF71 gene (Thome et al., 1997). The HIV nef protein inhibits Fas and TNF receptor mediated apoptosis of infected cells by blocking the apoptosis-signal-regulating kinase 1 (ASK1) (Geleziunas et al., 2001). Adenovirus, papilloma virus and HBV all target and inhibit the proapoptotic protein p53 (Benedict et al., 2002).

1.3.3 Counterattack

Rather than passive evasion of the host response, some viruses directly engage immune cells. The envelope glycoprotein (gp120) of HIV is involved in killing uninfected bystander T lymphocytes (Gougeon, 2005; Petrovas et al., 2005). HIV nef on the other hand induces the expression of Fas ligand (Fas L); this engages Fas on the non-infected CD8+ T cell compartment (that includes cells specific for the virus) and triggers them to apoptose (Xu et al., 1997). Additionally, immunosuppressive cytokines and chemokines or decoy receptors are used to counteract the immune response (Alcami, 2003). EBV encodes an interleukin 10 (IL-10) homologue that can inhibit the production of IFN-γ and TNF-α and decrease the expression of costimulatory molecules (ICAM-1, CD80 and CD86) (Jones et al., 2002; Kotenko et al., 2000; Spencer et al., 2002). Vaccina virus encodes vIFNa/bBP, a protein that binds to the cell surface and prevents type I IFNs from binding to its cognate receptors (Colamonici et al., 1995). Finally, some viruses decorate their external surface with immunomodulatory viral or host-derived proteins such as CD-family receptors, complement inhibitors, signalling ligands or adhesion molecules to achieve immunosuppressive activity (Cantin et al., 2005).

1.4 Hepatitis B virus epidemiology

The hepatitis B virus (HBV) belongs to the family hepadnaviridae that infects liver hepatocytes of birds, rodents, certain non-human primates and humans (Gould et al., 2001). First discovered in 1966, it is now one of the most important global pathogens. Two billion people (one third of the worlds population) have evidence of exposure to the virus and approximately 400 million have persistent infection (Mast et al., 1998); the latter group have a high risk of developing chronic hepatitis, cirrhosis, and hepatocellular carcinoma.

Global distribution can be divided into three areas: low, intermediate and high prevalence with <2%, 2-5% and >8% of individuals with chronic infection respectively. Within the latter, transmission occurs mainly at parturition with the risk of developing chronic infection estimated at 90%. This can be reduced to 10% if hepatitis B vaccination is administered at birth and the risk drops to below 5% if immunoglobulin to hepatitis B is also given (Beasley et al., 1983). From the age of 0 to 5 years horizontal infection, possibly via open wounds or contaminated injections, results in a 25-30% chronicity rate. This is partly due to prolonged viability of the virus outside the host (approximately one week) (Bond et al., 1981). Other horizontal routes of infection including sexual contact and drug use result in a 5-7% rate of chronicity in individuals from the age of 5 years upwards (McMahon, 2005).

1.5 HBV virology – genome

8 major genotypes, displaying >8% sequence diversity, have been defined (Locarnini, 2004). The viral genome is partially double stranded DNA and approximately 3181 to 3221 nucleotide pairs depending on the genotype. The circular configuration is maintained by a 226 base pair sequence overlap in between the 5' ends of the DNA strands that contain 11-nucleotide direct repeats referred to as DR1 and DR2. Once encapsidated, the minus strand of the genome maintains a fixed 5' and 3' end containing a terminal redundance of 8 to 9 nucleotides and the viral polymerase is covalently bound to the 5' end of the minus strand. This strand is not closed circular

but contains a nick near the 5' end of the plus strand. The 5' end of the plus strand is composed of an 18 base oligoribonucleotide which is capped in the same way as messenger RNA (Will et al., 1987). The 3' end of the plus strand varies in its position of termination; a gap is present that varies from between 20 to 80% of the viral genome and can be filled in by the endogenous viral polymerase. The viral genome minus strand contains four open reading frames (ORFs) that overlap by a frame shift. HBV is capable of producing multiple proteins from a single ORF by employing multiple initiation codons (AUG). The viral polymerase is encoded by the longest ORF. The envelope protein (PreS and S) ORF is contained within the ORF for Polymerase, and the ORF for the core (PreC/C) and X gene partially overlap with the Polymerase ORF (Locarnini et al., 2003) (figure 1.).

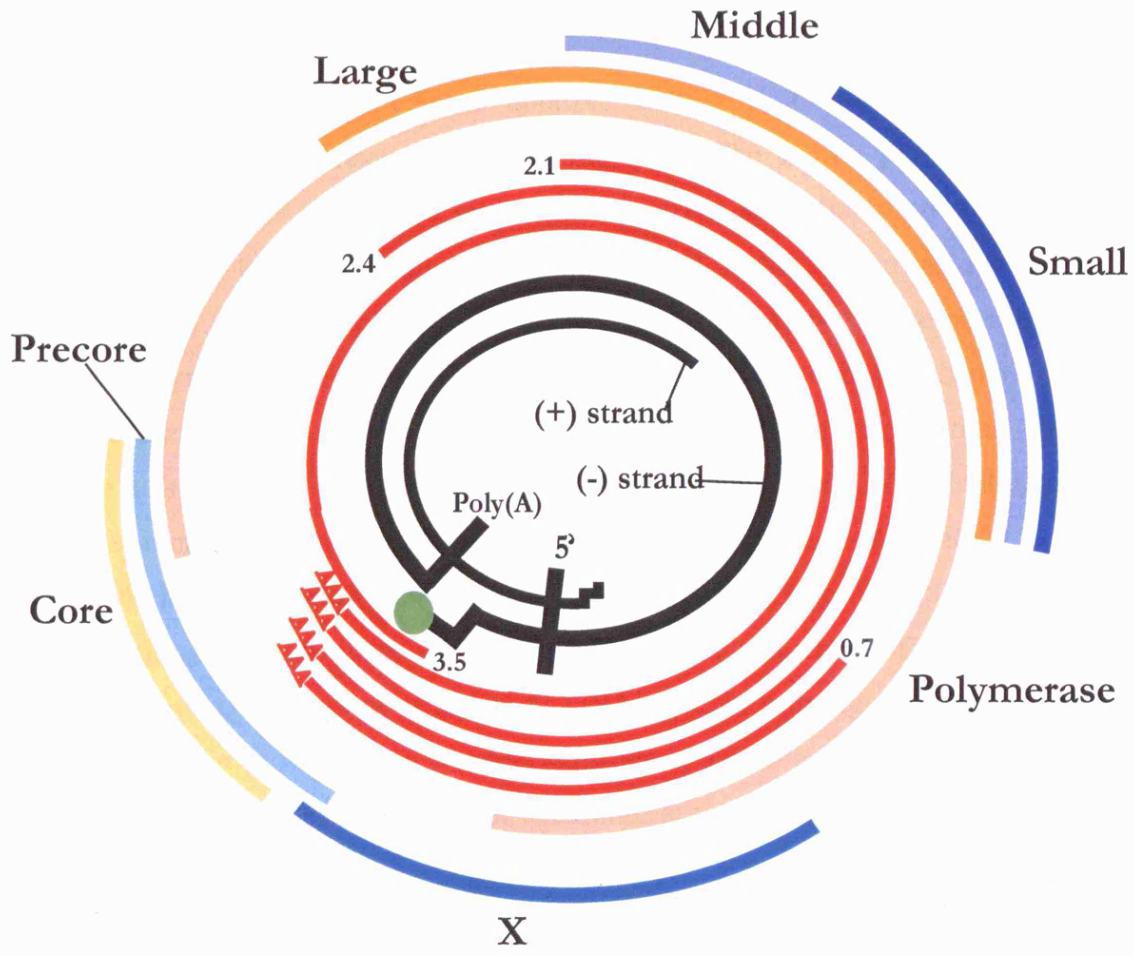

Figure 1.1: The genomic structure of the hepatitis B virus.

The inner black circles represent the full-length minus (-) strand (with the terminal protein attached to its 5' end; green spot) and the incomplete plus (+) strand of the HBV genome.

The red lines represent the 3.5, 2.4, 2.1 and 0.7 kilobase mRNA transcripts, which are all terminated near the poly(A) (polyadenylation) signal. The outermost coloured lines indicate the translated HBV proteins: large, middle and small HBV surface proteins, polymerase protein, X protein, and core and pre-core proteins.

Adapted by permission from Macmillian Publishers ltd. Nature Reviews Immunology; Rehermann, B; 2005; 5(3).

1.6 HBV virology - proteins

1.6.1 Polymerase

The polymerase protein spans approximately 80% of the viral genome and is translated from a pregenomic RNA molecule. The 90Kda protein product of the polymerase ORF contains at least 4 domains. The sequence in codons 834 to 845 is homologous to a reverse transcriptase. Domain 1, at the N terminal, forms a covalent linkage to the 5' end of the minus strand and is necessary for the priming of minus-strand synthesis. This is followed by a second spacer domain and then a third domain that performs the RNA- and DNA-dependent polymerase function. This region contains a YMDD motif that is essential for the RT activity. The final domain at the C terminal has ribonuclease H activity needed for cleavage of the RNA in the RNA-DNA hybrids during reverse transcription and is also involved in viral RNA packaging, optimization of the priming of minus-strand DNA synthesis, and elongation of the minus-strand viral DNA. Protein priming of reverse transcription relies on the provison of a tyrosine substrate at amino acid 63; this forms a covalent bond with the primary nucleotide (guanine) at the minus stand (Zoulim and Seeger, 1994).

1.6.2 Precore/Core

The Pre-C/C ORF encodes two proteins. The core protein is 183, 185 or 195 amino acids in length depending on the viral genotype and forms a coat that packages the viral genome. The ORF for core is preceded by a short upstream region that when transcribed in conjunction with core encodes for a protein referred to as HBe antigen (HBeAg).

HBcore (p21) is composed of two distinct domains: amino acids 1 to 144 are required for the assembly of the 32nm nucleocapsid and the second arginine rich domain

mediates nucleic acid binding, encapsidation and DNA replication. The arginine rich region further divides into four parts and contains a nuclear localization sequence.

HBe (p25) is a protein transcribed from precore/core mRNA and translates to a protein composed of the entire core but with an additional 29 amino acids at the N terminus. The first 19 amino acids form a signal responsible for its secretion into the lumen of the endoplasmic reticulum. Once there, these 19 amino acids are cleaved-off by a signal peptidase thereby producing a shorter protein, p22. p22 is modified by C–terminal cleavage of ~34 amino acids, resulting in a heterogenous array of protein products of 15 to 18 KDa long, defined as HBeAg. These can be secreted from the cell but not exclusively; a certain quantity remains within the cell. Contained within the p25 protein is a nuclear transport signal, thus HBeAg products are found in both nuclear and cytosolic compartments of the cell.

Although the primary sequences of HBeAg and HBcAg are similar, the final products differ significantly. HBeAg is essential to establishment of persistence. A woodchuck HBeAg-negative mutant was unable to establish persistence despite being infectious to newborn pups (Chen et al., 1992). Similar observations were made in human HBV infection (Milich et al., 1987). Additionally, HBeAg has been shown to function as a tolerogen *in vivo* (Milich et al., 1990). High levels of this protein are found in the serum during the tolerant phase of chronic infection alongside high levels of viral load.

There are a growing number of HBeAg seronegative individuals that contain a replication-competent viral mutant (commonly termed the precore mutant) that is associated with more severe disease outcome (Omata et al., 1991). The most common mutation is a nucleotide substitution A to T at position 1762 together with a G to A substitution at position 1764 resulting in a stop codon (TGG to TAG) at the end of the precore sequence (Kramvis and Kew, 1999).

1.6.3 PreS/S

Three sizes of HBsAg (small, medium and large) are utilized by the virus and occur in two forms that differ according to the extent of glycosylation. The secretion of infectious virions requires N-linked glycosylation as well as glucosidase processing. Subviral particles of 23nm diameter, as well as filamentous rods, (figure 1.3) lack viral genomes and do not require glycosylation for secretion.

The small HBsAg (SHBs), 226 amino acids in length, is the most abundant protein found in all three forms of secreted particles (subviral, rods and virions). This protein contains a high number of cysteine residues that cross-link with each other forming a loop that is a major antigenic determinant previously referred to as the "a" determinant but now renamed the major hydrophilic region (MHR) located between amino acids 99 to 160 (Carman et al., 1990). The subdeterminants d, y, w and r contain a lysine, arginine, lysine or arginine at position 122, 122, 160 and 160 respectively.

The medium HBsAg (MHBs; PreS2) is made of the SHBs coupled to a 55 amino acid N-terminal region and is a minor component of the secreted particles. The protein can be single or double glycosylated but neither form is required for viral assembly and secretion. The central region contains the MHR and a region between amino acids 3 and 16 has the ability to bind to polymerised serum albumin however, a role for this interaction has not as yet been demonstrated. MHBs has greater immunogenic properties than SHBs with respect to B cell responses (Milich et al., 1985).

The large HBsAg (LHBs; PreS1) is composed of 108 or 119 amino acids coupled to the N-terminal of the MHBs protein. It occurs in a great excess compared to MHBs in both virons and filaments, but less so in subviral particles. In a mature viral particle, the PreS domains are exposed at the surface but shielded by the PreS1 portion of the LHBs. This protein is essential for infection of target cells via a myristylated N terminal region.

1.6.4 X protein

The X protein (HBxAg) is 154 amino acids in length and 17Kda in size and although it is not required for viral production (*in vitro*) it is critical for infectivity *in vivo*. HBxAg protein functions as a transcriptional transactivator of various viral and cellular gene promoters by direct interaction with transcription factors and also participates in the activation of signal transduction pathways (Zoulim and Seeger, 1994). HBxAg also acts as a co-factor in the development of hepatacellular carcinoma possibly through alterations in cellular gene expression (Rossner, 1992). Additionally, interaction with and inactivation of the tumour suppressor p53 has been demonstrated (Truant et al., 1995); p53-dependent apoptosis can be interrupted by HBxAg (Wang et al., 1994). Finally, HBxAg has been shown to target the 26S proteosome complex thus interfering with the efficiency of protein processing and presentation that could in turn affect the recognition by the hosts T lymphocyte compartment (Hu et al., 1999).

Figure 1.2: The three forms of hepatitis B surface antigen.

Electron Micrograph of HBV by Linda Stannard, Department of Medical Microbiology, University of Cape Town. The virus is 42nm in diameter and possesses an isometric nucleocapsid or "core", surrounded by an outer coat. The protein of the virus coat is termed "surface antigen" or HBsAg. It is sometimes extended as a tubular tail on one side of the virus particle. The surface antigen is generally produced in vast excess, and is found in the blood of infected individuals in the form of filamentous and spherical particles. Filamentous particles are identical to the virion "tails"; they vary in length and have a mean diameter of about 22nm. http://web.uct.ac.za/depts/mmi/stannard/hepb.html.

1.7 Hepatitis B virus replication

The initial event of the life cycle involves attachment of the virus to the target cell. Amino acids 21-47 of the PreS1 domain are required for hepatocyte binding as demonstrated *in vitro* with the HepG2 cell line (Neurath et al., 1986); binding was blocked by antibodies to preS1 (amino acids 21-47). The PreS2 domain is not required for entry however it may enhance viral uptake and uncoating. Its ability to bind human serum albumin suggests that it may act as a bridging moiety; hepatocytes express a receptor for human serum albumin. SHBs can also bind primary hepatocytes, however the significance of this is not well understood. Ultimately, virus binding to the appropriate receptor (which has not been identified although several have been proposed (Neurath et al., 1986) allows delivery of the nucleocapsid to the cytoplasm. This is transported to the nucleus where the viral genome is converted into cccDNA.

cccDNA formation involves filling in of the gap region, removal of the 5'-terminal structures and covalent ligation of the two strands. The cccDNA is organized into nucleosomes which forms the viral minichromosome that is the major template for the transcription of the two classes of viral mRNA; subgenomic and genomic. All species are capped and polyadenylated. The former are translated into envelope and X protein, while the latter produce precore, core and polymerase. Pregenomic mRNA has two functions: it serves as a template for the translation of core and polymerase proteins and it is used for the production of the DNA negative strand by reverse transcription. The latter occurs primarily within core particles, the formation of which is initiated by the binding of polymerase to a pregenomic mRNA transcript. Capsids containing the relaxed circular DNA are enveloped only after reverse transcription has been initiated to a point at which a signal is transmitted to the surface of a capsid. Enveloped particles are deposited into the lumen of the endoplasmic reticulum, move to the golgi apparatus and are finally secreted from the cell (figure 1.3). Subviral particles are formed from the aggregation of approximately 100 HBsAg monomers in the endoplasmic reticulum.

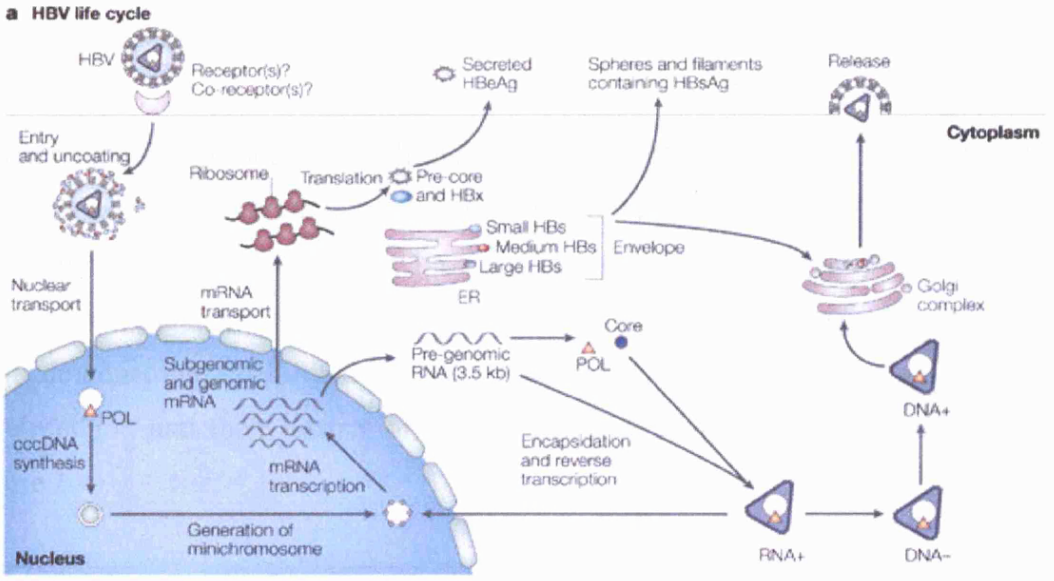

Figure 1.3: Putative life cycle of HBV.

After entry to the cell, hepatitis B virus (HBV) nucleocapsids transport their cargo, the genomic HBV DNA, to the nucleus, where the relaxed circular DNA is converted into covalently closed circular (ccc) DNA. The cccDNA functions as the template for the transcription of four viral RNAs (of 0.7 kilobases (kb), 2.1 kb, 2.4 kb and 3.5 kb), which are exported to the cytoplasm and used as mRNAs for the translation of the HBV proteins. The longest (pre-genomic) RNA also functions as the template for replication, which occurs within nucleocapsids in the cytoplasm. Nucleocapsids are enveloped during their passage through the endoplasmic reticulum (ER) and/or Golgi complex and are then secreted from the cell.

Adapted by permission from Macmillian Publishers ltd. Nature Reviews Immunology; Rehermann, B; 2005; 5(3).

1.8 Natural history of HBV infection

1.8.1 Acute resolving infection

Primary infection of susceptible hosts is either symptomatic or asymptomatic, with the latter being more common, particularly in children. Although one percent develop fulminant hepatitis associated with coagulopathy, encephalopathy and cerebral oedema, probably due to an excessive immune response, the majority of healthy adults demonstrate self-limited infection that is associated with long-lasting immunity to reinfection and their history of infection can be divided into three main stages (figure 1.4).

Stage one corresponds to a period of active viral replication associated with immune tolerance lasting up to four weeks. HBeAg, HBsAg and infectious virions can normally be detected in the serum. In the majority, active viral replication ensues without a significant elevation in serum aspartate and alanine aminotransferase (ALT). Amino-L-transferase, a protein normally found within hepatocytes, is released into the circulation when these cells are damaged and is used as a measure of liver integrity; approximately 35 and 45 IU/ml are considered normal for females and males respectively.

Stage two is associated with the generation of a significant immune response that results in a dramatic reduction in the serum viral load; maximal viral suppression occurs before significant liver damage most likely though noncytolytic antiviral mechanisms (Guidotti et al., 1999; Webster et al., 2000). The subsequent rise in serum ALT indicates the initiation of liver damage; symptomatic hepatitis lasts for approximately three to four weeks manifesting as fever, jaundice and weight loss. Viral replication in infected hepatocytes is subsequently controlled, resulting in the loss of serum HBeAg and the appearance of anti-HBeAg antibodies. HBsAg can still be detected possibly due to integration of the HBsAg gene into the hepatocyte genome. Individuals remain sero-negative for antibody to HBsAg. Serum HBV DNA

is greatly reduced to very low levels that can be detected by PCR but not hybridization techniques and serum ALT levels returns to normal.

In the last stage individuals become HBsAg negative and anti-HBsAg antibody positive; HBV DNA is undetectable by PCR indicating successful viral containment.

1.8.2 Chronic infection

Although, the majority of healthy adults manage to contain the virus, ~5% do not resolve but instead maintain chronic infection. This manifests as an extremely heterogenous disease (see figure 1.4b).

Primary infection of neonates and children is subclinical and anicteric; approximately 90% of infants of HBsAg and HBeAg-positive mothers become carriers (McMahon et al., 1985) compared to 30% of children infected between 1 to 5 years (Chang, 2000). A high maternal HBV DNA and HBeAg level is associated with increased risk of developing chronic infection (Burk et al., 1994) that is characterized by a prolonged immunotolerant phase and a low rate of HBeAg clearance (Chang, 2000). However, less than 10% of infants born to mothers that are HBeAg negative and anti-HBeAg antibody positive develop chronicity (Chang, 2000). Perinatal infection is characterized by a prolonged immunotolerant phase with a low rate of spontaneous HBeAg clearance; entry into the immunoactive phase, 10 to 30 years later, is associated with the development of chronic hepatitis with elevated ALT (Lok et al., 1987). Those that were infected during childhood present in the immunoactive phase earlier in life; the majority have raised ALT and seroconvert to an anti-HBeAg antibody positive status sooner than those infected perinatally, usually during adolescence (Fattovich, 2003).

30 to 50% of adults that experience primary infection develop icteric hepatitis (McMahon et al., 1985) of which 1 to 5% maintain chronic infection (Tassopoulos et al., 1987). Some of these exhibit high viral loads, typically 10^7 to 10^9 virions/ml

(Weinberger et al., 2000) associated with HBeAg seropositivity - apart from those that have precore mutations. Each year 5-10% of individuals with high viral load suppress viral replication by up to five orders of magnitude, clear HBeAg from their circulation and develop anti-HBeAg antibody; this is often preceded by a transient rise in salt, also termed a flare (Ribeiro et al., 2002).

The other group of chronically infected adults maintain a long-term inactive carrier state; anti-HBeAg antibody positive, HBeAg seronegative, with relatively low levels of viral replication compared to the acute phase. The majority sustain biochemical remission and very low risk of cirrhosis and hepatocellular carcinoma (de Franchis et al., 1993; Hsu et al., 2002). 20 to 30% undergo spontaneous reactivation of high-level HBV replication and multiple episodes can lead to progressive liver damage and hepatic decompensation (Perrillo, 2001). 1 to 2% of inactive carriers resolve infection (develop antibody to HBsAg and loose HBsAg) (Alward et al., 1985; Fattovich et al., 1998).

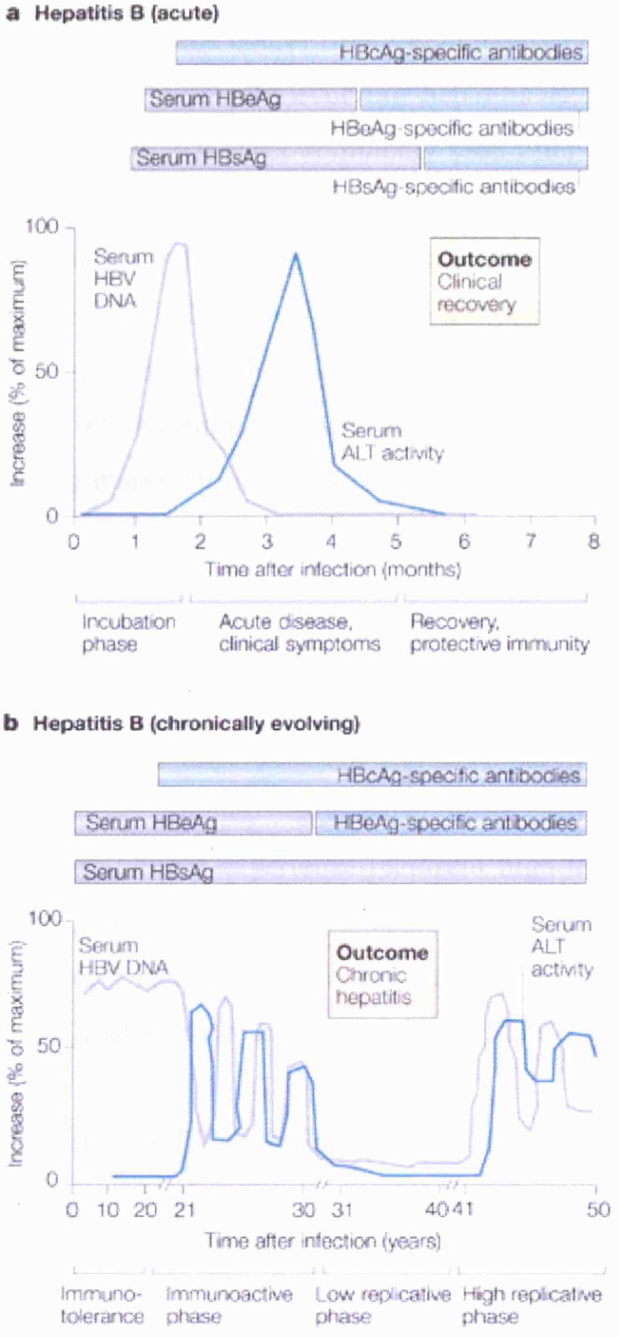

Figure 1.4: Clinical and virological course of acute and chronic hepatitis B virus (HBV) infection.

(a) A schematic depiction of the immune response in acute infection with hepatitis B virus (HBV) through horizontal transmission, followed by clinical recovery, is

shown. After recovery, neutralizing HBV surface antigen (HBsAg)-specific antibodies and HBV-specific T cells confer lifelong, protective immunity (for further details, see main text). (b) Chronically evolving hepatitis B results from vertical transmission. Chronic hepatitis B is most commonly seen after vertical transmission from mother to neonate. The course of disease is characterized by several phases of variable length. The immunotolerant phase is characterized by high levels of circulating HBV DNA and HBV e antigen (HBeAg) and normal alanine aminotransferase (ALT) levels, and this phase can last for decades. For unknown reasons, it can transition into an immunoactive phase, in which HBV DNA titres are lower but liver disease is markedly more severe and can progress to liver cirrhosis. Alternatively, the immunoactive phase might transition into a low replicative phase, with clearance of free HBeAg from the serum and development of HBeAg-specific antibodies. In the low replicative phase, serum HBV DNA is typically below the detection limit of hybridization assays; ALT levels also normalize, and necroinflammatory liver disease improves. The low replicative phase might last for life, but a subgroup of patients, especially those who have undergone immunosuppressive therapy, might experience recurrent high-level HBV replication and marked necroinflammatory liver disease. Mutations in the promoter region of the gene that encodes HBV core antigen (HBcAg), which are associated with increased replication, and pre-core mutations, which result in an HBeAg-negative phenotype, have been described.

Adapted by permission from Macmillian Publishers ltd. Nature Reviews Immunology; Rehermann, B; 2005; 5(3).

1.9 Host immunity to viral infection

The specialized cells of the immune system derive from common pluripotent self-renewing haematopoetic stem cells (CD34+) located in the bone marrow (Lessard et al., 2004) that differentiate into myeloid or lymphoid progenitors. Myeloid progenitors further differentiate into the various cells of the innate immune compartment and the lymphoid progenitors differentiate into natural killer cells, dendritic cells and the two categories of cells of the adaptive immune compartment, the B and T lymphocytes (Galy et al., 1995).

1.10 Innate host immunity to viral infection

The innate immune compartment is composed of non-clonal cells whose main function is to stop the progressive infection of susceptible cells and to raise a state of alert. It is particularly critical at the early stage of infection. The response is immediate and does not result in selective memory. Effector cells include neutrophils, basophils, eosinophils, granulocytes, monocytes, dendritic cells, and natural killer and natural killer T cells. While some of these denature the pathogen following endocytosis, others engage with and subsequently secrete a variety of antimicrobial inflammatory cytokines and chemokines. In addition to these specialized cell types, epithelial cells and endothelial cells are also able to participate in the innate response through the immediate production of cytokines, chemokines and antimicrobial peptides following the recognition of pathogens (Andonegui et al., 2003; Eckmann et al., 1997; Hertz et al., 2003; Kagnoff and Eckmann, 1997).

1.10.1 DC subsets

Dendritic cells are the professional antigen presenting cells of the body; they capture antigen, migrate to local draining lymph nodes, and present antigen-derived peptides to naïve or quiescent memory T lymphocytes. They are capable of activating cells with the cognate TCR, thereby driving their proliferation into effector cells (Jung et al., 2002; Norbury et al., 2002; Smith et al., 2003); these expanded T lymphocyte populations exit the lymph node and guided by chemokines, migrate to the inflamed site where they exert cytolytic/noncytolytic function. Dendritic cells themselves are a heterogenous population that have been broadly divided into six subsets. The first main division is between plasmacytoid DCs and the other five DCs subsets (Grouard et al., 1997); the latter can be grouped according to the site from where they are derived, either blood or tissue.

1.10.1.1 Plasmacytoid DCs

Plasmacytoid DCs are found throughout the body but are not thought to be important for antigen presentation. Their main role during the induction phase of the immune response appears to be the production of type I IFNs (Cella et al., 1999; Diebold et al., 2003) following TLR-mediated recognition of pathogens (Asselin-Paturel et al., 2001; Kadowaki et al., 2001; Nakano et al., 2001). This leads to the inhibition of viral replication as well as the activation of other DCs, particularly those specializing in cross-presentation (Le Bon et al., 2003). This subset can express CD8α particularly following activation (Henri et al., 2001).

1.10.1.2 Tissue derived conventional DCs

Lymph nodes that drain the skin contain two tissue-derived DCs: langerhans cells (Romani et al., 2003) and dermal interstitial DCs. Langerhans cells are found in the epithelia of the skin and act as sentinels. Pathogen encounter induces migration via

draining lymphatics to lymph nodes. Recent evidence indicates that these cells may not actually prime CD8+ T cells (Allan et al., 2003; Zhao et al., 2003) but may instead cater more to the transportation of antigens (Allan et al., 2006; Carbone et al., 2004; Randolph, 2006; Villadangos and Heath, 2005; Yoneyama et al., 2005). Organs other than the skin do not contain langerhans cells but have DCs that are similar to dermal interstitial DCs. This indicates that each organ contains its own specific interstitial DC subset and potentially raises the total number of subsets to over six. A distinguishing feature of tissue-derived DCs is the expression of the mannose receptor CD205 (Henri et al., 2001). This marker is also present on CD8+ DCs but the subsets can be easily differentiated from each other because the latter express the CD8+ coreceptor at a high level.

1.10.1.3 Blood-derived conventional DCs

Blood-derived conventional DCs can be separated according to their expression of CD4 and CD8 coreceptors (Vremec et al., 2000; Vremec et al., 1992); they are either double negative (CD4-CD8- DCs), CD4+ (CD4 DCs) or CD8+ (CD8+ DCs). Double negative DCs preferentially produce IFN-γ (Hochrein et al., 2001); no dominant cytokine production has been associated with CD4 DCs. CD8+ DCs are thought to be the main immunoactive subtype in the blood: they are the predominant IL-12 producers (Steinman et al., 1997) and have a central role in cross-presentation (den Haan et al., 2000).

1.10.2 Role of DCs in HBV

Although it is known that infection with persistent viruses such as HIV and HCV can be associated with impaired DC function (Auffermann-Gretzinger et al., 2001; Donaghy et al., 2003; Kanto et al., 1999; Steinman et al., 2003a), the role of DCs in HBV infection has only recently been studied and is under debate. Tavakoli *et al* have reported that HBV infection of (monocyte-derived) DCs lead to phenotypic and

functional alterations but the antiviral T cell stimulatory capacity of these cells was maintained (Tavakoli et al., 2004). On the other hand, Van der Molen *et al* have demonstrated that chronic HBV infection is associated with the functional impairment of both myeloid and plasmacytoid DCs (van der Molen et al., 2004). Additionally, Untergasser *et al* have recently shown that although DCs were capable of taking up viral particles, the microbe did not replicate within these cells (Untergasser et al., 2006) suggesting that contact with circulating viral antigen particularly subviral particles, rather than infection by virus, could be contributing to altered DC functionality.

1.10.3 Innate recognition of pathogens

There are two main categories of pathogen recognition: extra and intra-cytoplasmic (Meylan and Tschopp, 2006).

1.10.3.1 Extracytoplasmic sensing of pathogens

The extracytoplasmic pathway for pathogen sensing relies on several pattern-recognition receptors (PRRs) such as C-type lectins, scavenger receptors, pentraxins, lipid transferases, integrins, and leucine-rich proteins that include the Toll-like receptor family; these sample the extracellular environment for pathogen-associated molecular patterns (PAMPs) (Medzhitov and Janeway, 1997). The TLR family (particularly TLR3, 7, 8 and 9) has received considerable attention because of their relevance to infection with viruses; upon engagement with cognate ligand, these molecules transmit signals through cytoplasmic Toll/Interleukin-1 receptor (TIR) domains resulting in the transcriptional induction of multiple genes, particularly those for type I IFNs (figure 1.5).

Toll like receptor 3 (TLR3) is expressed on the cell surface and recognizes extracellular double stranded RNA (dsRNA) released following lysis of infected

cells. Engagement triggers a series of cellular events that culminate in the activation of the transcription factors IFN regulatory factor 3, neural factor kB (NF-KB), and activating protein 1 (AP1) that coordinate the transcriptional regulation of the IFN-β gene (Kawai and Akira, 2006; Sasai et al., 2006; Wathelet et al., 1998). TLR3 signalling has been shown to be particularly important for the cross-presentation of soluble antigen by CD8+ DC to CD8+ T cells (Fujimoto et al., 2004).

TLR 7, 8 and 9 are located in the endosomes of plasmacytoid DCs (Kato et al., 2005). Viral particles or infected apoptosing cells are endocytosed thereby facilitating the recognition of PAMPs by N-terminal endosomal leucine-rich repeats. Fc receptor mediated endocytosis of virus-antibody complexes can also lead to TLR triggering (Wang et al., 2007). Subsequent intracellular signalling through the MyD88-IRAK1/4-IRF1/3/7 pathway culminates in the expression and release of IFN α & β (Kawai and Akira, 2006; Marie et al., 1998).

TLR7 and TLR8 recognize ribonucleic acid homologs and ssRNA rich in guanosine or uridine (Heil et al., 2004; Lund et al., 2004) (Diebold et al., 2004) typical of HIV-1 and influenza genomes. TLR9 on the other hand recognizes unmethylated 2'-deoxyribo cytidine-phosphate-guanosine (CpG) DNA motifs present in viral DNA. Synthetic CpG oligonucleotides are potent promoters of T helper type I responses and have recently been shown to improve responses to HBV vaccines particularly in low-responders (Cooper et al., 2005; Cooper et al., 2004; Verthelyi et al., 2004).

1.10.3.2 Cytoplasmic sensing of pathogens

The intracellular (cytoplasmic) recognition of viruses occurs through retinoic acid-inducible gene I (RIG-1) and melanoma differentiation-associated gene 5 (Mda5) (figure 1.9). These proteins belong to a DExD/H box RNA helicase family that have, in addition to a helicase domain, an N-terminal caspase recruitment domain (CARD). They function by sensing viral RNA through the helicase and then transmit a signal through the CARD domain (Meylan et al., 2005) that leads to the activation of

transcription factors IRF3, NK-kB and AP-1 that in turn induces IFN-b. Apart from recognizing dsRNA (Kato et al., 2006; Yoneyama et al., 2004) Rig-1 is also activated by ssRNA that contain 5'-phosphates (Pichlmair et al., 2006). Studies have also shown that the HCV protein NS3/4A protease abrogates RIG-1 mediated antiviral function by cleaving a cofactor (mitochondria-associated Cardiff protein) that is required to initiate IFN production (Breiman et al., 2005; Vilasco et al., 2006).

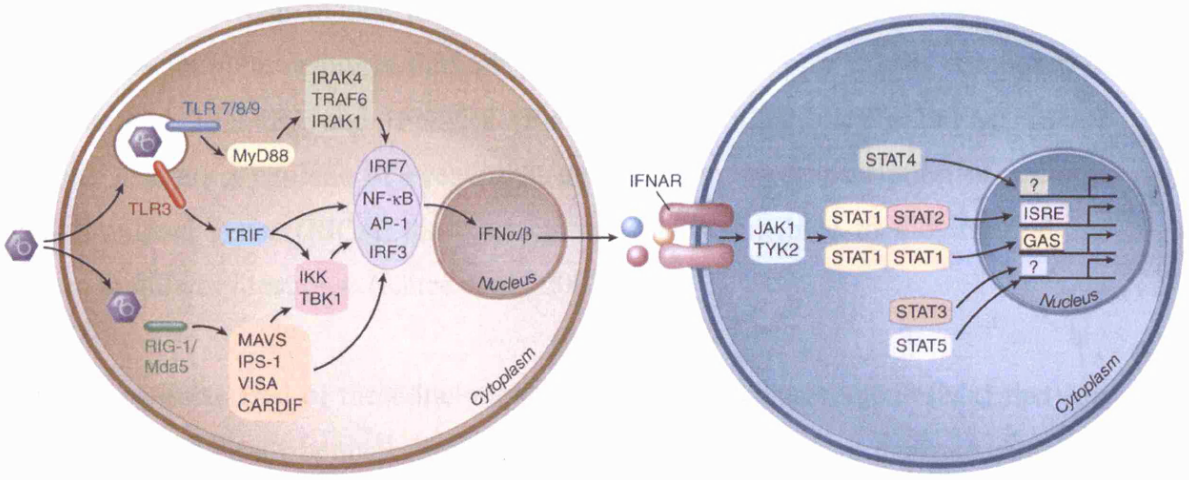

Figure 1.5: The pathways to and from type I interferons.

Families of sensors are available to detect viral products and induce expression of cytokines. One set senses components in the cytoplasm. Another set is localized in cell membranes. There is also flexibility in the signaling pathways used by type 1 IFNs, with the potential to induce the activation of multiple STAT molecules and their downstream targets of transcription.

Adapted by permission from the American Association for the advancement of Science. Garcia-Sastre, A; 2006: 312

1.10.4 Type 1 Interferons

Type 1 Interferons (IFNI) are composed of multiple genes clustered within one locus on chromosome 9 (Theofilopoulos et al., 2005); humans have 13 subfamiles of IFN-α and only one IFN-β gene (Weissmann and Weber, 1986). They can be divided into an immediate early response group (IFN-α4 and IFN-β) and a late group (IFN-α2, 5, 6&8); the former can induce the latter (Marie et al., 1998). All of these proteins regulate the immune response through the type 1 IFN receptor that is composed of two subunits, IFNAR1 and IFNAR2 (Platanias, 2005) (Brierley and Fish, 2002). Binding triggers a cascade of events that culminate in the transcription of multiple IFN-stimulated genes (ISGs), the products of which contribute to the antiviral and immunoregulatory response (Katze et al., 2002).

The best characterised of these include the myxovirus resistance gene (Mx) that acts by sequestering viral ribonucleoproteins to specific subcellular compartments; the 2'-5' oligoadenylate synthetase (OAS) that generates 2'-5' oligoadenylates which activate ribonuclease L (RNase L) leading to the degradation of cellular and viral RNA; and the dsRNA-stimulated serine-threonine protein kinase (PKR) that, following recognition of dsRNA, phosphorylates downstream substrates such as the elongation initiation factor 2A (eIF2A) leading to an inhibition of translation. The latter of these has been implicated in the 10-fold reduction of nucleocapsids containing pregenomic RNA and reduction in viral replication in HBV-transgenic mice (Guidotti et al., 2002; Wieland et al., 2000).

Recently, APOBEC3G, a member of the apolipoprotein B mRNA-editing catalytic polypeptide-like editing complex (ABOBEC) superfamily of cytidine deaminases that have antiviral activity against a variety of viruses (Harris et al., 2003; Mangeat et al., 2003), has also been shown to be upregulated by IFN-α resulting in a decrease in HBV replicative intermediates (Rosler et al., 2005).

1.10.5 Natural Killer (NK) cells

NK cells are a distinct subset of lymphoid cells that have innate immune functions (Cerwenka and Lanier, 2001) and exert antiviral activity in a non-MHC dependent manner. They are activated by cytokines or following encounter with target cells that express ligands for NK receptors (Lanier, 2005); the outcome of NK activity is determined by a balance of signals from activating and inhibitory receptors. Particular inhibitory receptors recognize MHC class I molecules that are normally present on all healthy cell types. In addition to host-derived or pathogen-induced activation signals, down-regulation of MHCI following infection is the additional danger signal that triggers NK activity (Ljunggren and Karre, 1985).

NK cells are capable of both cell-to-cell contact-dependent cytolysis and the production of high levels of cytokines. Type I and II IFNs, IL-12 and IL-15 are thought to be required for optimal NK activity against viral infections (Lee et al., 2000; Nguyen et al., 2002a). NK cells have been shown to be critical for the inhibition of HBV replication *in vivo* (transgenic mouse model) (Kakimi et al., 2000); the robust activation of IFN-γ and TNF-α that immediately follows the exponential phase of HBV infection (Guidotti et al., 1999) is likely to derive from these populations that normally account for one third of total intrahepatic lymphocytes (Sprengers et al., 2005).

Recent work in acutely infected chimpanzees has demonstrated that that the rapid decrease in viral replication in resolving chimps occurs in conjunction with intrahepatic production of IFN-γ prior to the recruitment of T lymphocytes (Guidotti et al., 1999). Similarly, in a human study during the incubation phase of HBV infection, suppression of viral replication was also associated with an increased circulating frequency of NK cells that preceeded the appearance of HBV-specific CD8+ T cells (Webster 2000). Our own studies indicate that the cytokine-enhanced antiviral activity of NK cells could be contributing to the immunopathology that is observed in chronic HBV infection (Dunn et al., 2007).

1.11 Adaptive immunity to viral infection

The adaptive immune compartment exerts specific resistance to infection that is clonal and exhibits memory upon repeated exposure to the original antigen. It is composed of two main compartments: B and T cells.

1.11.1 B Lymphocytes

B cells develop from a lymphoid progenitor that differentiates within the bone marrow. These cells recognize conformational epitopes; antigens that are either present on the surface of infected cells or secreted into the extracellular environment. Each naïve B cell expresses a unique immunoglobulin receptor on its cell surface. Following recognition of antigen these cells undergo three main events. They begin to secrete large quantities of soluble immunoglobulin into the circulation; this is aimed at neutralizing free circulating antigen. These cells also undergo somatic hypermutation to modify the reactive immunoglobulin in order to recognize the antigen better. Third, following internalization and processing of the antigen, they migrate to secondary lymphoid organs where they function as professional antigen presenting cells that prime T cells. Type I and type II interferons bridge the gap between innate and adaptive immunity by regulating the development, proliferative potential, immunoglobulin secretion and Ig heavy chain switching capacity of B cells (Schroder et al., 2004; Stark et al., 1998).

The B cell response to HBV envelope antigens (HBsAg) is T cell-dependent (Milich and McLachlan, 1986); these antibodies are readily detectable in resolved individuals but not present in chronic infection. Viral containment occurs by the formation of antibody-virus complexes preventing attachment and entry into hepatocytes. The antibody response to HBcAg and HBeAg is not fully understood; it does not neutralize viral infectivity because it can be present alongside high titres of virus, however, chimpanzees that have passively been administered HBeAg antibodies were protected from HBV infection (Stephan et al., 1984). It also appears that the anti-

HBe/cAg antibody response can function in a T cell-independent manner because this antibody response can be strong in chronic infection when the T cell response is known to be weak (Milich and McLachlan, 1986). The antibody response to HBV polymerase and X protein has not been well studied.

1.11.2 T lymphocytes

T lymphocytes develop from lymphoid progenitors that leave the bone marrow as double negative thymocytes and migrate via the blood to the thymus where they differentiate into T helper (CD4+) or T cytotoxic (CD8+) cells.

1.11.2.1 Central Tolerance

In each day of the postnatal period of life, approximately 10 to 100 hematopoiteic progenitors enter the thymus from the bone marrow via the bloodstream. Over the course of two weeks, double negative thymocytes undergo approximately 20 divisions (Porritt et al., 2003; Shortman et al., 1990) resulting in the production of about 50 million T cells/day of which only 1 to 2 million exit the thymus (Kyewski and Klein, 2006). More than 95% of the cells are lost. The initial process, β selection, relies on pre TCR signalling and allows only double negative (DN; CD4-CD8-) with successfully rearranged TCRβ genes to proceed to the double positive status (CD4+CD8+) (von Boehmer et al., 2003) upon which TCR rearrangement occurs. Once the αβ receptor is expressed the T cell it is subject to series of TCR/peptide/MHC interactions that determine its survival. A large proportion of T cells cannot bind MHC alleles of the host. The process of positive selection in the cortex of the thymus selects for those that can recognise self-peptide-MHC complexes, permitting only these to further differentiate. This step, also termed death by neglect, accounts for the majority (90-95%) of cell death. It also indicates that the preference for selection of self-specificity exceeds the selection against autoreactive cells.

About 50-70% of these cells are subsequently subjected to negative selection that occurs in the medulla region of the thymus and is exerted principally by thymic dendritic and thymic epithelial cells (Derbinski et al., 2001; Hanahan, 1998). This latter population are able to express peripheral antigens and pass them to thymic dendritic cells (DCs) to increase exposure – reports estimate the DCs scan ~ 5000T/hour (Miller et al., 2004). The promiscuous expression of peripheral antigens, as well as late self-antigens, extends the scope of central tolerance. In this way T cells with high avidity to self-peptide/MHC, and thus capable of inducing autoimmunity, are neutralized. Low avidity T cells proceed unhindered towards maturation and are allowed to exit the thymus and populate secondary lymphoid tissues.

1.11.2.2 Peripheral Tolerance

Not all self-specificities are eradicated within the thymus; although most self-proteins are expressed at this site (Su and Anderson, 2004), some specificities do manage to escape. In order to avoid the induction of autoimmune responses to the latter, T cells subsequently encounter peripheral tolerance mechanisms. Under specific conditions, peripheral TCR/self-peptide/MHC interactions result in the induction of unresponsiveness (anergy) or activation induced cell death (peripheral deletion).

Apart from a TCR-dependent stimulus, T cells require the provision of costimulatory signals. Costimulatory proteins are upregulated on professional antigen presenting cells (pAPCs) during inflammation or infection thereby allowing T cells to distinguish between noninfectious-self and infectious-self (Medzhitov and Janeway, 2000). Thus, T cells stimulated by pAPCs that do not have appropriate (raised) levels of costimulatory proteins are anergised (Macian et al., 2004; Schwartz, 2003); the hallmarks of which are the inability to proliferate, secrete cytokines and differentiate into functional effectors (DeSilva et al., 1991).

A large number of co-stimulatory receptors have been described (Bertram et al., 2004b; Croft, 2003; Kroczek et al., 2004) (figure 1.6). The first subgroup, the

immunoglobulin superfamily, includes CD28 and the inducible costimulator (ICOS), of which the former is better characterized (Sharpe and Freeman, 2002); the respective ligands include B7.1 & B7.2 and ICOS ligand (that is related to the B7 family) (Sharpe and Freeman, 2002). CD28 signalling is required and in fact sufficient for the induction of optimal activation, maintaining viability and preventing anergy in naïve and resting cells (Harding et al., 1992; Jenkins et al., 1991); ICOS signalling on the other hand is more relevant to the costimulation of previously activated and effector cells. The immunoglobulin family also includes co-inhibitory receptors (PD-1 and CTLA-4); these are normally upregulated following successful activation of the cell and are designed to maintain overall T cell homeostasis by silencing the specific response (Greenwald et al., 2005). The second group, the tumour necrosis factor receptor (TNFR) superfamily includes OX40, 4-1BB, CD27, CD30 and HVEM, the ligands for which are OX40L, 4-1BBL, CD70, CD30L and LIGHT (Croft, 2003).

In addition to DCs, the liver contains other antigen presenting cells. Liver sinusoidal endothelial cells (LSECs) and Kupffer cells are able to present antigen to CD4+T cells but fail to induce differentiation to a Th1 phenotype (Knolle et al., 1999); instead, these cells produce IL-10 and TGF-β that downregulate T cell activation thereby promoting anergy (Knolle et al., 1998) or apoptosis of T cells (Knolle and Gerken, 2000).

The precise mechanism of anergy induction is thought to involve the cell cycle inhibitor p27Kip1 that interferes with the cyclin-dependent kinase 2 (CDK2) and the maintenance of the activity of the transcription factor Smad3 (Li et al., 2006) (figure 1.7). Suboptimal activation due to substandard costimulation fails to downregulate p27Kip1 resulting in an impaired proliferation; p27Kip1 –deficient cells are resistant to anergy (Rowell et al., 2005), whereas the upregulation of this protein induces anergy and tolerance despite the provision of costimulation (Boussiotis et al., 2000a; Powell et al., 1999).

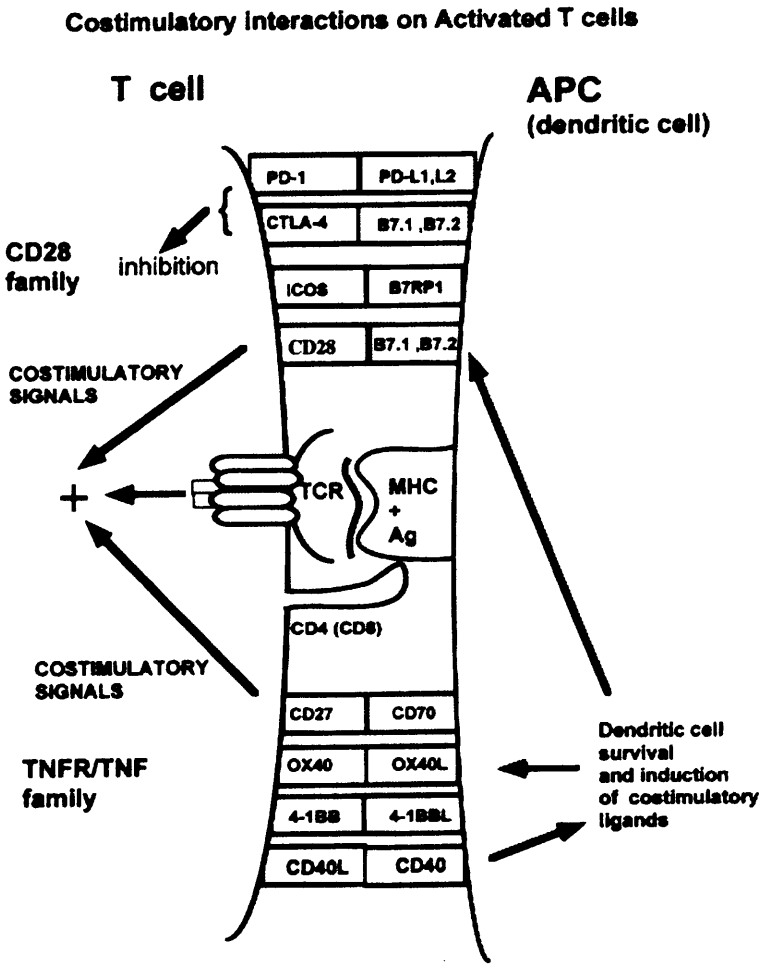

Figure 1.6: Costimulatory and inhibitory interactions on activated T cells.

The CD28 superfamily includes the stimulatory members CD28 and ICOS and the inhibitory members, CTLA-4 and PD-1. CD40L a member of the TNF ligand family is expressed on activated T cells, and its binding to CD40 on dendritic cells induces expression of 4-1BBL, OX40L and CD70, the ligands, respectively, for 4-1BB, OX40 and CD27. Although these interactions are illustrated as taking place on the same cell, in vivo, they may involve different subsets of APC and T cells, different locations in the lymphoid organ and may also be temporally segregated. Based on expression of TNF/TNFR ligands other TNF/TNFR interactions could involve T–T, T–B, T–NK, NK–B, NK–DC interactions and could involve TNFR receptor expression on APC as well as T cells.

Adapted by permission from Elsevier Ltd. Seminars in Immunology; Bertram, EM; 2004: 16(3).

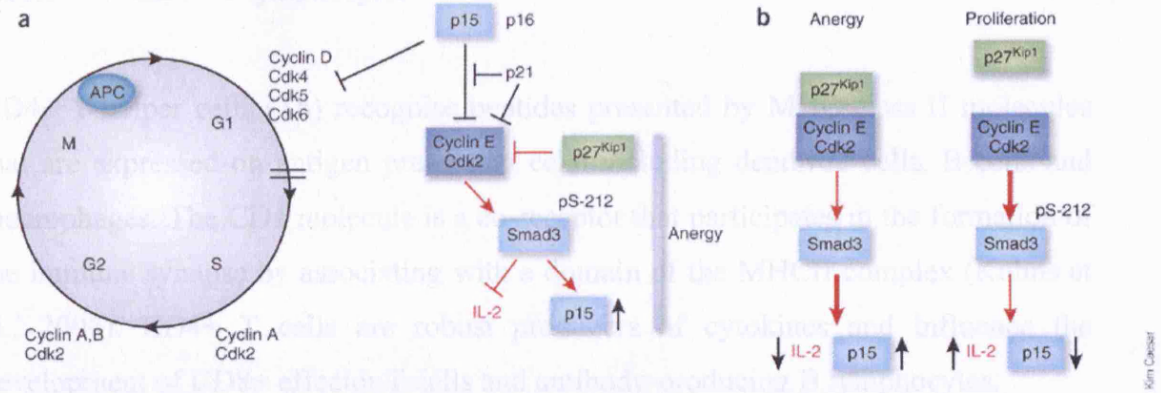

Figure 1.7: The tolerogenic pathway involves inhibition of Cdk2 activity by p27Kip1 and an increase in Smad3 function.

(a) Regulation of the cell cycle. Cells progress from G0 or G1 to S (DNA synthesis), G2 and mitosis. Cyclin E–Cdk2 complexes drive the transition from G1 to S, whereas cyclin A–Cdk2 and cyclin A–cyclin B–Cdk2 complexes are involved in S and G2, respectively. The anaphase-promoting complex (APC) is required for anaphase and exit from mitosis. The inhibitor p27kip1 binds and inhibits cyclin E–Cdk2 kinase, whereas Cdk2 can phosphorylate the serine at position 212 of Smad3 (pS-212) and inhibit its transcription activity. Smad3 increases p15 (Cdk inhibitor 2B) and inhibits IL-2 production. The proteins p15, p16CdkN2a (p16; also called INK4a) and p21Waf1/Cip1 (p21) can inhibit Cdk4 needed for G1-phase progression. (b) In this model, anergy and in vivo tolerance is induced by p27Kip1 binding and the inhibition of cyclin E–Cdk2 complexes, which leads to less inhibitory phosphorylation of Smad3. That results in increased Smad3 activity, increased p15 expression and inhibition of IL-2 production. Conversely, proliferation and clonal expansion results from a loss of p27Kip1 expression and/or binding to cyclin E–Cdk2, leading to an increase in inhibitory phosphorylation of the serine at position 212 of Smad3 and a reduction in p15 and upregulation of IL-2 production.

Adapted by permission from Nature Publishing Group. Rudd, CE; 2006: 7(11)

1.11.3 CD4+ T lymphocytes

CD4+ T helper cells (Th) recognise peptides presented by MHC class II molecules that are expressed on antigen presenting cells including dendritic cells, B cells and macrophages. The CD4 molecule is a co-receptor that participates in the formation of the immune synapse by associating with a domain of the MHCII complex (Kuhns et al., 2006). CD4+ T cells are robust producers of cytokines and influence the development of CD8+ effector T cells and antibody-producing B lymphocytes.

Quiescent naïve CD4+ T cells circulate between secondary organs via the blood and lymphatics (Girard and Springer, 1995) surveying the body for appropriate triggers. Priming follows TCR/peptide/MHCII engagement, costimulation, and signals from the surrounding cytokine/chemokine milleu (Molon et al., 2005). This results in a series of rapid divisions that are associated with the acquisition of the capacity to secrete effector cytokines (Murphy and Reiner, 2002). Fully differentiated daughter cells then migrate to appropriate sites where they secrete specific cytokines to functionally organize the immune response.

Several subsets of CD4+ T cells have been defined according to their cytokine secreting profile (figure 1.8). T helper (Th) 1 and Th2 cells (produce IFN-g and IL-4 respectively; however, Th 0 cells produce both of these cytokines) (Lund et al., 2005; Mosmann and Coffman, 1989), Th3 cells (transforming growth factor β) (Carrier et al., 2007a, b), Th follicular helper cells (ThFH) (Chtanova et al., 2004; Rasheed et al., 2006), Th17 (IL-17-A) (Acosta-Rodriguez et al., 2007; Harrington et al., 2005), T regulatory (Tr) 1 cells (IL-10) (den Haan et al., 2007) and peripherally-induced T regulatory (Treg) cells (FoxP3+) (Maloy and Powrie, 2001) (Reinhardt et al., 2006; Ruprecht et al., 2005).

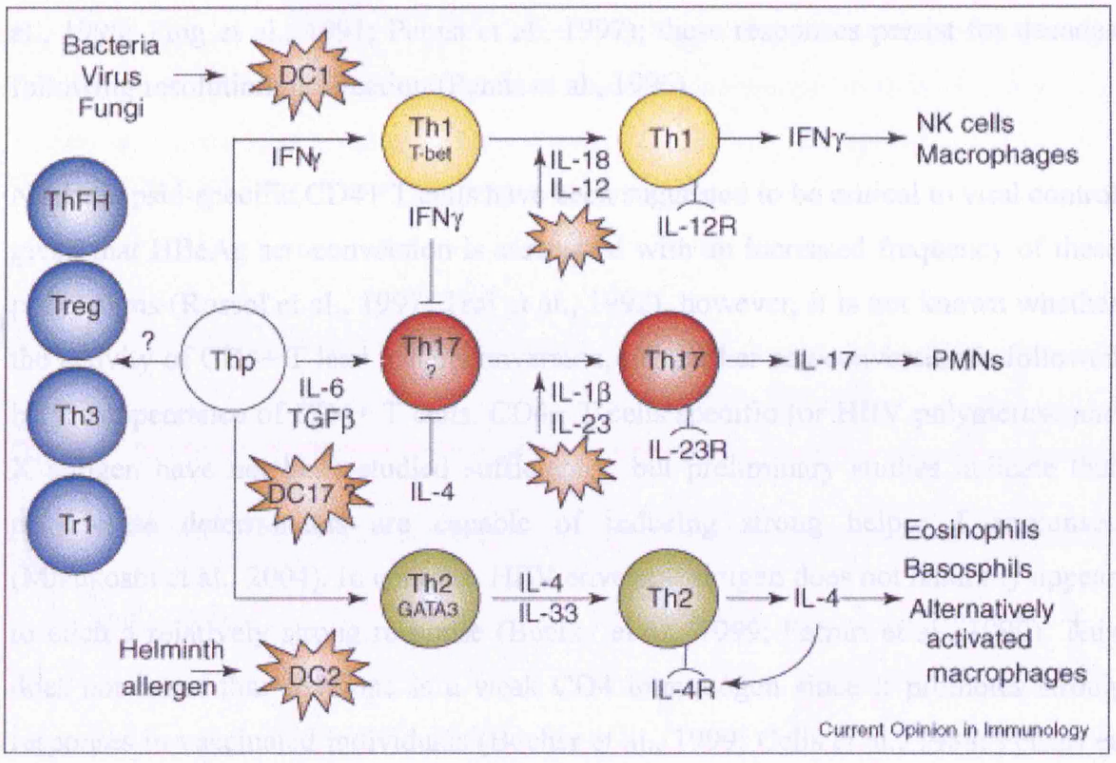

Figure 1.8: T helper subsets.

Although the precise lineage of the subsets in blue has not been defined by cell fate-mapping experiments, Th1, Th17 and Th2 cells represent alternative effector cell fates individually derived from naïve precursor T cells (Thp). The degree to which these cells fates are conditioned by different classes of dendritic cells (DC) is unknown. The differentiation, maintenance and effector functions of these subsets share certain symmetries. Th1 cells express T-bet and IL-12 receptors (IL-12R) downstream of IFNγ signals, and are further maintained by IL-12 and IL-18, members of the greater IL-6-like and IL-1-like superfamilies, respectively. Th17 cells develop in the presence of TGFβ and IL-6, and are further maintained by IL-23 and IL-1β, members of the same superfamilies. Th2 cells are maintained by IL-4 itself, as well as by the IL-1 superfamily member IL-33. Downstream, Th subsets recruit distinct innate cell types to mediate effector functions in the periphery.

Adapted by permission from Elsevier Limited. Current Opinion in Immunology; Reinhardt RL; 2006:18(3).

Self-limited HBV infection is associated with Th1 CD4+ T that recognise multiple determinants in the core protein and respond by producing IL-12 & IFN-γ (Ferrari et al., 1990; Jung et al., 1991; Penna et al., 1997); these responses persist for decades following resolution of infection (Penna et al., 1996).

Nucleocapsid-specific CD4+ T cells have been suggested to be critical to viral control given that HBeAg seroconversion is associated with an increased frequency of these populations (Rossol et al., 1997; Tsai et al., 1992), however, it is not known whether the activity of CD4+ T lead to seroconversion, or whether seroconversion is followed by the appearance of CD4+ T cells. CD4+ T cells specific for HBV polymerase and X antigen have not been studied sufficiently, but preliminary studies indicate that polymerase determinants are capable of inducing strong helper T responses (Mizukoshi et al., 2004). In contrast, HBV envelope antigen does not naturally appear to elicit a relatively strong response (Bocher et al., 1999; Ferrari et al., 1990). This does not mean that envelope is a weak CD4 immunogen since it promotes strong responses in vaccinated individuals (Bocher et al., 1999; Celis et al., 1988; Ferrari et al., 1989); this however, could be attributed to differences in antigen presentation that are related to the use of synthetic adjuvants.

The influence CD4 T regulatory cells during HBV infection is not well understood. Some studies have demonstrated an increased frequency of Tregs in the peripheral circulation of individuals with chronic HBV infection (Barboza et al., 2007; Fu et al., 2007; Stoop et al., 2005; Xu et al., 2006). These data are in conflict with other work (Franzese et al., 2005); although depletion of Tregs increased the function of the HBV-specific T cells, this was observed in chronic as well as resolved infection indicating that the mechanism was not specific to infection with HBV.

IL-10 appears to be a critical factor in influencing the protective immune response to viral infections (Klenerman and Ludewig, 2006) and can determine progression to either chronicity or effective control (Brooks et al., 2006b). Blockade of the IL-10 receptor has been shown to contribute to resolution of chronic infection (Ejrnaes et al., 2006). HIV-1 and HCV-specific T cells have been shown to exhibit IL-10

mediated immunosuppressive activity (Accapezzato et al., 2004; Elrefaei et al., 2007). IL-10 secreting HBV-specific T cells have also been described recently (Hyodo et al., 2004) and allude to Tr1 mediated immunosuppressive mechanism. In line with these findings, our own studies have revealed a significant elevation of IL-10 in the serum of patients with chronic infection, compared to resolvers (unpublished observations; Maini, MK *et al.*, UK.).

1.11.4 CD8+ T lymphocytes

CD8+ T lymphocytes recognise peptides in the context of MHC class I molecules that are ubiquitously expressed by all nucleated cells and are critical to the control of intracellular pathogens. Infected cells are recognised via the TCR. The majority of T cells (both CD4+ and CD8+) bear receptors that are composed of an α and a β chain. γδ TCR receptors predominate earlier on in ontogeny but occur at relatively smaller frequencies from birth onwards apart from localised sites such as the gut where they exhibit tolerogenic activity (Chien and Konigshofer, 2007; Kapp et al., 2004).

1.11.4.1 TCR α chain

The α chain consists of a variable (V) amino-terminal region, joining (J) segments and a constant (C) region whereas. The C region encodes a transmembrane polypeptide and is involved in anchoring the TCR to the cell surface. It also participates in intracellular signal transduction pathways. The V, J and C gene segments are rearranged by somatic recombination during T cell development in the thymus with additional diversity introduced by the protein TdT that catalyses the addition of nucleotides at the gene segment junctions. Heptamer and nonamer recombination signal sequences flank the gene segments and are recognized by a complex of enzymes called VDJ recombinase. Lymphoid-specificity is conferred from products of RAG-1 and Rag-2 (recombination-activation genes) genes. Gene

arrangement results in the excision of gene segments that take the form of T cell receptor excision circles (TRECs) (Ye and Kirschner, 2002).

1.11.4.2 TCR β chain

The β chain consists of variable (v), diversity (d), joining (j) and constant (c) gene segments and undergoes rearrangement before the alpha chain. Dβ segments rearrange to Jβ segments, followed by vβ to DJβ. Survival of the thymocyte depends on the expression of a pre-T cell receptor. Thus, if the rearranged construct does not produce a functional β chain, the VDJ arrangement has the potential of being rescued by subsequent rearrangements from two additional clusters of Dβ and Jβ gene segments that occur upstream of the two Cβ genes. When a productive β chain arrangement is finally achieved it is expressed in conjunction with an invariant partner chain (pTa) and CD3 molecules and transported to the surface of the cell. This expression signals the phosphorylation and subsequent degradation of Rag-1 and Rag-2, thereby terminating further β chain gene rearrangement. Cell proliferation ensues, followed by the expression of CD4 and CD8+ allowing the production of multiple double positive thymocytes with identical β chains but still lacking the α chain.

Once proliferation is terminated, the Rag genes are expressed once again and the α chain segments begin to undergo specific rearrangement and expression culminating in an αβ TCR thymocyte. Rearrangement of the α chain can however continue despite production of a TCR at the cell surface. Several α chains are produced successively and simultaneously within each developing T cell and are tested, in conjunction with a consensus β chain, for recognition of self-antigen. This phase of gene rearrangement is terminated upon positive selection.

The D and J segments of the α and β chains contribute to the formation of a hyper variable loop termed the complementarity determining region 3 (CDR3) which forms the center of the peptide binding site of TCR. Diversity is concentrated in this region

that is surrounded by the CDR1 and CDR2 loops that are encoded by the less variable (v) segments that make contact with the (relatively less variable) MHC molecule. The TCR variable loops fits diagonally across the peptide-MHCI binding site and buries most of the peptide as well as a large part of the MHC (Garboczi et al., 1996).

1.11.4.3 The CD3 complex

The TCR α and β chains do not possess enzymatic activity nor do they undergo covalent modifications by intracellular enzymes. The TCR is used to transmit signals though nonpolymorphic polypeptides that make-up the CD3 complex; these include γ, δ, ε and disulphide linked ζ chain dimers (Samelson et al., 1985; Weissman et al., 1986). Following TCR ligaton, conformational changes induce the phosphorylation of tyrosine residues in the CD3 polypeptides; one site on γ, δ, αvδ ε and three on each ζ chain (Samelson et al., 1986). Phosphorylation, catalysed by two cytosolic Src family kinases (Lck and Fyn), occurs on 16 amino acid long consensus sequences termed immunoreceptor-based tyrosine activation motifs (ITAMs) that bind to the SyK-family tyrosine kinase, ZAP-70 (Chan et al., 1992; Wange et al., 1992). Differences in ζ chain phosphorylation are though to differentially activate the Lck and Fyn kinases that in turn recruit particular adapter proteins to transmit a the signal that either leads to productive T cell activation or anergy. Lck kinase activity has been associated with productive activation whereas Fyn has been associated with anergy (Boussiotis et al., 1996; Gajewski et al., 1995; Gajewski et al., 1994).

1.11.4.4 The CD8 coreceptor

CD8 is a disulphide-linked heterodimer that consists of an α and a β chain each of which contains an immunoglobulin-like domain that is linked to the membrane by an extensively glycosylated segment - this segment serves to maintain the polypeptide in an extended conformation and it also protects the molecule from protease cleavage. When the CD8 α chain is expressed without the β chain it is able to form

homodimers that are involved in alternative specific functions – non-classical MHCI interactions. CD8 ($\alpha\beta$) on the other hand interacts with the invariant region of the $\alpha 3$ domain of the MHCI molecule and the strength of this interaction is related to the extent of glyscolyation - an increase in the proportion of sialic acid residues decreases the strength of the interaction. The variation of sialic acid composition varies with maturation and activation state. The cytoplasmic tail of the CD8 a chain on the other hand binds lck, a src- family tyrosine kinase, and brings it into closer proximity with the TCR.

1.11.4.5 The Major Histocompatibility Complex Class I

Almost all nucleated cells have MHCI, and these complexes present peptide fragments of endogenously expressed proteins. This is critical to protection of the host because it allows the immune system to detect cells that are infected by pathogens. In the case of viral infections, viral proteins that are synthesized as part of the viral replication cycle are processed and fragments of these polypeptides are displayed on the surface of the cell.

The human MHC Class I [termed the human leukocyte antigen (HLA)] was first defined serologically with alloantibodies in the 1950s. The structure consists of two polypetide chains. The larger of the two, the α chain, is encoded by a genetic locus located on chromosome 6 and contains a carboxy terminal transmembrane spanning region. Three classes exist, denoted A, B and C. Their highly polymorphic nature contribute to their broad peptide binding specificity. A smaller non-polymorphic second chain, β microglobulin, encoded on a separate chromosome (15 in humans) is non-covalently attached and completes the dimer.

The completed molecule is composed of 4 domains, three of which are produced by the α chain and the fourth is produced by β microglobulin. The A3 domain and B2-microglobulin fold together to form an immunoglobulin structure. The $\alpha 1$ and $\alpha 2$ domains form a cleft on the surface of the molecule that is able to bind short peptides

usually 8 to 10 amino acids in length. Longer peptides are also able to settle into the cleft particularly if the carboxy terminus suits binding, but the extended bits are normally subsequently digested off by exopeptidases in the endoplasmic reticulum.

Peptide binding in the MHC molecule cleft is stabilized by contacts at both ends between atoms in the amino and carboxy termini of the peptide and the invariant sites at the end of the cleft with the peptide lying in an elongated conformation along the cleft. These contact points are the main stabilizing contacts. Peptides that bind to a specific MHC variant also contain the same (or similar) aminio acid residues at two or three specific positions. The residues at these locations insert into pockets in the MHC and are known as anchor residues. Not all peptides that conform to anchor residue requirements are able to bind a given MHC variant; binding is reflective of the precise composition and electrostatic nature of the individual amino acids (Falk et al., 1991); certain amino acids may be preferred at secondary sites (Barouch et al., 1995).

HLA-A2, the first human type I major histocompatability complex to be defined, is present at a high frequency in most populations (Browning and Krausa, 1996). The structure of HLA-A2 was obtained through X-ray crystallography in 1985 (Bjorkman et al., 1987a, b) and a consensus HLA-A2-binding peptide motif was subsequently identified by sequencing peptides eluted molecules; 9mer peptides with anchor residue leucine and valine/leucine are required at position 2 and 9 respectively (Falk et al., 1991).

1.11.5 The CD8+ T cell response

A successful CD8+ T cell response to a pathogen can be divided into four phases (figure 1.9):

Phase 1 begins with the priming of naïve CD8+ T cell by mature antigen presenting cells such as dendritic cells, macrophages or B cells (Lanzavecchia and Sallusto, 2001). An optimal response requires the provision of three signals. Signal 1 is delivered through the TCR following engagement with the peptide/MHCI complex on the pAPC, signal 2 is provided by costimulatory molecules also on the pAPCs, and signal 3 is provided by the inflammatory cytokines IL-12, Type I and Type II IFNs (Haring et al., 2006). Additionally, the formation of a stable interaction has been shown to greatly influence the outcome; a 4-hour stimulus resulted in abortive clonal expansion, whereas a longer (20-hour) engagement leads to an effective response (Schoenberger, 2003).

Phase 2 covers the expansion of successfully activated CD8+ T cells and lasts for approximately 5 to 8 days; in this time the frequency of antigen-specific cells increases greater than 10,000-fold (Badovinac and Harty, 2002; Kaech et al., 2002). Cells undergo up to 3 divisions a day and can divide between a minimum of 8 and up to 15 times before they arrive at their peak (Kaech and Ahmed, 2001; Selin et al., 1996). This phase also involves subsequent differentiation into a population of cells with poor proliferative capacity but strong functionality and migratory capacity that are able to travel throughout the body and home to infected cells (Weninger et al., 2002).

Phase 3 involves the elimination of 90-95% of the expanded cells and occurs over approximately one week (Sprent and Tough, 2001). The kinetics of the contraction phase is independent of the dose, duration of infection or amount of antigen that is presented, rather, it appears that the early programming events during and immediately subsequent to CD8+ T cell priming govern the fate of the cell

(Badovinac et al., 2002; Mercado et al., 2000; van Stipdonk et al., 2001; Wong and Pamer, 2001).

The final phase of the response involves the maintenance of a memory pool composed of the cells that have survived phase 3 (Kaech et al., 2002). These populations are able to respond faster to antigen re-encounter thereby providing more effective immunity (Bachmann et al., 1999a; Bertram et al., 2004a; Kersh et al., 2003; Slifka and Whitton, 2001). Additionally, the ability to express CD25 has been shown to be critical for the development of secondary responses emphasizing the importance of help during the early priming events (Kalams and Walker, 1998; Williams et al., 2006).

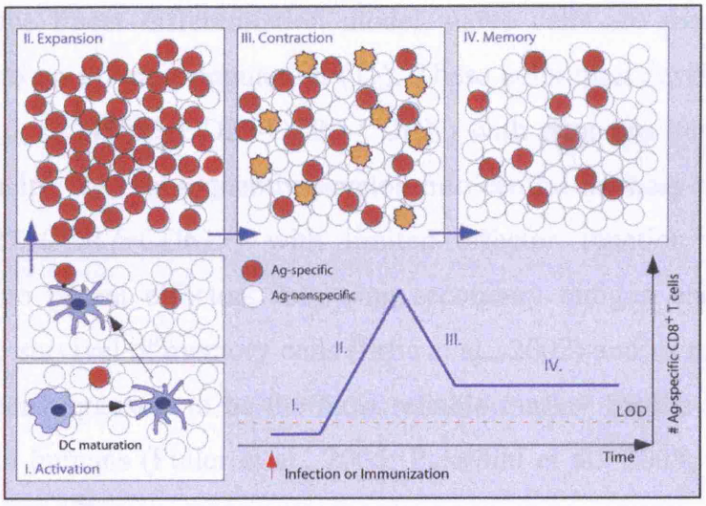

Figure 1.9: Naive-to-memory CD8+ T cell progression after acute infection or vaccination.

Antigenic peptides presented by mature dendritic cells (DCs) to naive CD8++ T cells trigger expansion and differentiation into effector CD8++ T cells. Five to ten percent of CD8+ T cells detected at the peak of expansion survive the contraction phase to initiate the memory pool, which can remain stable in number for life. LOD represents limit of detection

Printed with permission from Elsevier Limited. Immunity; Haring, JS.; 2006: 25(1)

1.11.6 CD8+ T cell differentiation

The differentiation of CD8+ T cells has been well described and subsets have been delineated according to their homing capacity and effector function (Masopust et al., 2001; Reinhardt et al., 2001; Sallusto et al., 2004; Sallusto et al., 1999).

According to the linear differentiation model, naïve cells are thought to initially differentiate into an effector population (T_E). These cells then develop into effector memory T (T_{EM}; CD45RO+CD27-CCR7-CD62-) with poor proliferative but potent functional capacity. T_{EM} subsequently develop into central memory population (T_{CM}; CD45RO+CD27+CCR7+CD62+) with limited effector function but capable of proliferation into potent effectors following secondary antigen encounter. IL-7 is required for the survival of memory cells (Prlic et al., 2002) and its receptor (CD127) has recently been suggested to be the most reliable marker for the identification of memory cells in humans (Fuller et al., 2005; Paiardini et al., 2005; van Leeuwen et al., 2005) (figure 1.10).

The progressive differentiation model on the other hand favours the involvement of an intermediate T subset (T_{im}) that develops from naïve cells that can then directly give rise to either T_{EM} or T_{CM} (Bachmann et al., 2005a; Bachmann et al., 2005b; Huster et al., 2006) (figure 1.11).

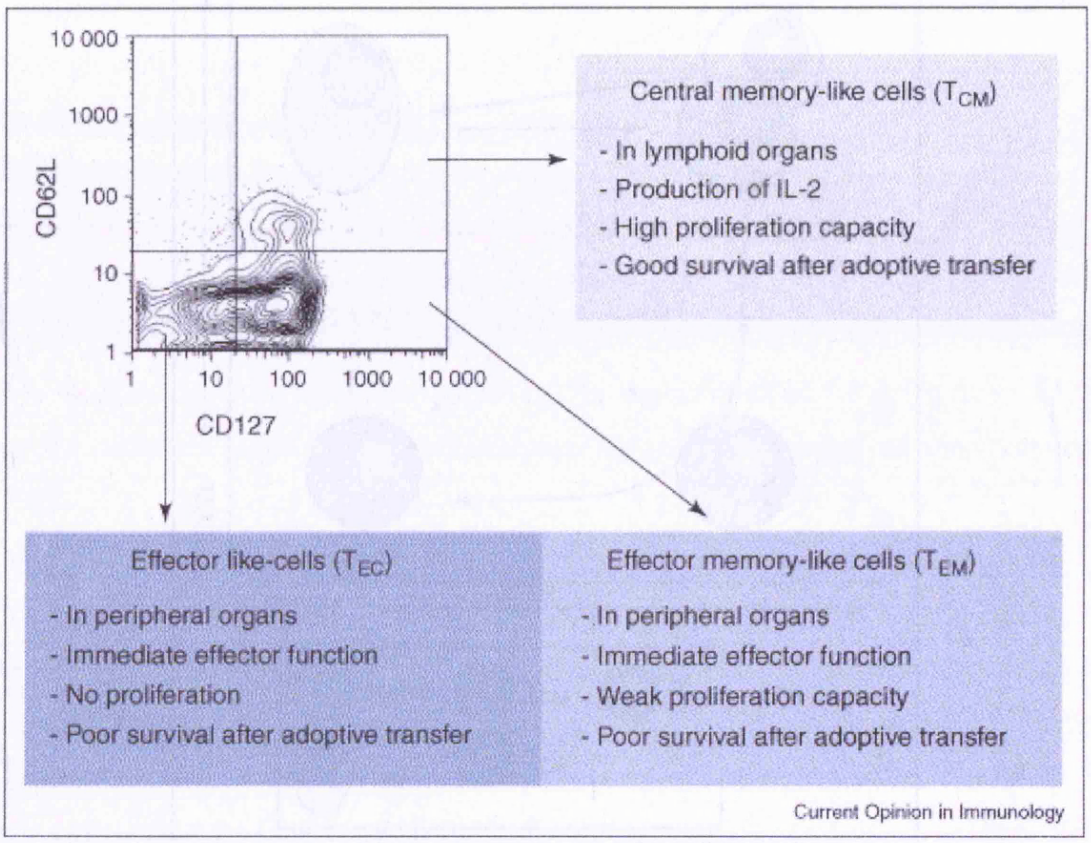

Figure 1.10: Correlation between phenotype and function of antigen-specific CD8+ T cells.

Adapted by permission from Elsevier Limited. Current Opinion in Immunology; Huster, KM; 2006: 18(4).

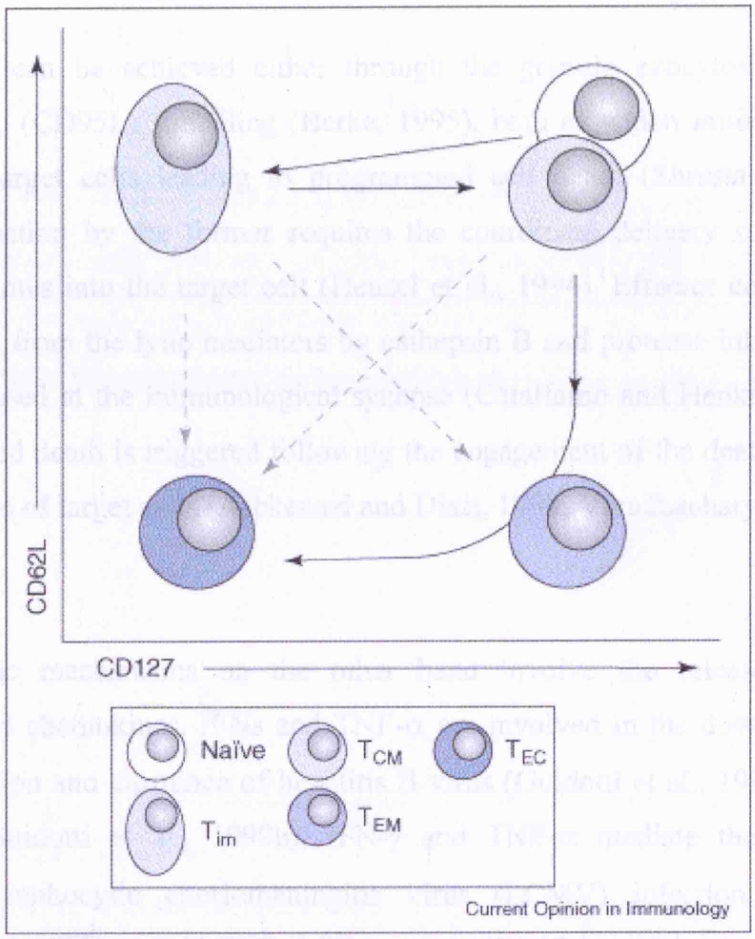

Figure 1.11: Progressive differentiation model.

Proposed lineage relationship of CD8+ T cell subsets. Dashed lines indicate potential differentiation pathways, which have so far not been elucidated.

Adapted by permission from Elsevier Limited. Current Opinion in Immunology; Huster, KM; 2006: 18(4).

1.11.7 CD8+ T cell antiviral function

Although commonly referred to as cytotoxic T lymphocytes (CTL), CD8+ T cells are able to exert antiviral function through both cytotoxic and non-cytotoxic mechanisms.

Cytotoxicity can be achieved either through the granule exocytosis pathway or through FasL (CD95L) signalling (Berke, 1995), both of which initiate the caspase cascade in target cells leading to programmed cell death (Shresta et al., 1998). Efficient function by the former requires the coordinate delivery of perforin and granule enzymes into the target cell (Heusel et al., 1994). Effector cells themselves are protected from the lytic mediators by cathepsin B and protease inhibitor 9 (PI-9) that are released at the immunological synapse (Catalfamo and Henkart, 2003). Fas ligand induced death is triggered following the engagement of the death receptor Fas on the surface of target cells (Ashkenazi and Dixit, 1998; Varadhachary and Salgame, 1998).

Non-cytotoxic mechanisms on the other hand involve the release of antiviral cytokines and chemokines. IFNs and TNF-α are involved in the downregulation of gene expression and clearance of hepatitis B virus (Guidotti et al., 1994; Guidotti et al., 1996; Guidotti et al., 1999b), IFN-γ and TNF-α mediate the clearance of persistent lymphocytic choriomeningitis virus (LCMV) infection of the liver (Guidotti et al., 1999a); RANTES, MIP-1α and MIP-1β have been implicated in the control of HIV-1 infection (Cocchi et al., 1995). The downstream pathways remain to be properly defined, however two IFN-induced pathways have been recently been proposed and are thought to act by shutting-off viral protein synthesis (Schneider and Mohr, 2003; Tian and Mathews, 2001).

Persistent stimulation of CD8+ T cells can result in a sequential loss of effector function ultimately leading to deletion (Wherry and Ahmed, 2004).

1.11.8 CD8+ T cell response to HBV

CD8+ T cells are the principal effector cells that contribute to viral containment (Guidotti et al., 1996); this has been clearly demonstrated in CD8+ T cell depletion studies in a chimpanzee model (Thimme et al., 2003). Studies have shown that an effective HBV–specific CD8+ T cell response is robust and multispecific (Bertoletti et al., 1991; Jung et al., 1999; Maini and Bertoletti, 2000; Maini et al., 2000a; Maini et al., 1999; Penna et al., 1991). Both cytolytic and noncytolytic mechanims are involved (Guidotti et al., 1999; Murray et al., 2005). The main CD8+ T cell effector cytokines, IFN-γ and TNF-α, mediate viral clearance by destablizing the nucleocapsid an degrading viral proteins and HBV RNA (Biermer et al., 2003). The role of this arm of the adaptive immune response is covered in more detail in chapter five.

1.11.9 CD8+ T cell homeostasis

The principal function of activated T cells is to exert effector function that contributes to the control of pathogens. This mechanism must be tightly controlled; a careful balance that is neither autoimmune pathology at one extreme nor immunodeficiency at the other must be achieved (Arnold et al., 2006). Two separate pathways govern the fate of activated cells: an active activation induced cell death (AICD) and a passive activated T cell autonomous death (ACAD) (Lenardo et al., 1999) (figure 1.12).

T cell undergo AICD as a consequence of signalling through cell surface death receptors receptors (figure 1.13) whereas ACAD is the result of an intrinsic mechanism that involves the balance of pro- and anti-apoptotic factors that are influenced by TCR signalling and/or cytokine deprivation (figure 1.14). Both pathways converge into caspase activation.

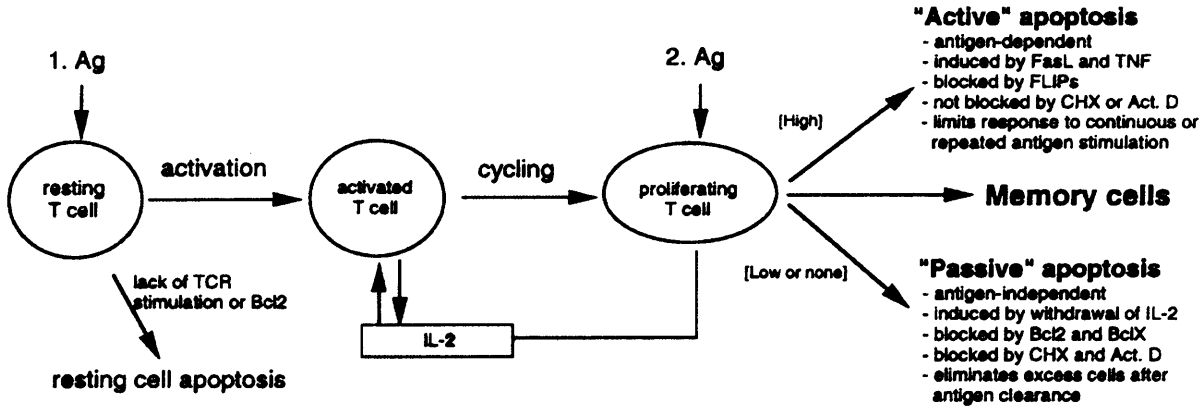

Figure 1.12: The propriocidal or feedback-response paradigm of mature T lymphocyte apoptosis.

The T cell response to antigen occurs in two molecularly distinct phases: activation, which leads to the production of IL-2; and proliferation, which is due to cell cycle progression caused by IL-2. Cycling T cells become highly susceptible to apoptosis and whether death ensues depends on the environmental conditions. Active apoptosis occurs if strong secondary TCR engagement is encountered. Passive apoptosis occurs after cessation of antigen and IL-2 stimulation. A small number of cells escape both death pathways and these are believed to become the "memory" population.

1.11.9.1 Extrinsic apoptotic pathway

The tumor necrosis factor receptor family are a group of proteins that contain a death domain in the cytoplasmic component of the polypeptide. Ligation of the appropriate ligand to the receptor induces clustering which in turn recruits adaptor proteins that also contain a death domain such as FADD and TRADD (Boldin et al., 1995a; Boldin et al., 1995b; Chinnaiyan et al., 1995; Hsu et al., 1995). Fas ligand binds and oligomirizes Fas (CD95/Apo-1) which in turn allows recruitment of FADD. Other receptors require the intermediate adaptor TRADD before FADD can be positioned appropriately. FADD can then recruit procaspase-8 and procaspase-10 via a DED interaction (Boldin et al., 1996; Muzio et al., 1998). This increases their activity and they are then able to autoactivate (Martin et al., 1998; Muzio et al., 1998) thereby continuing the apoptosis chain of events. Additionally, cell surface death receptors can lead to events other than apoptosis. TNFR1 ligation can activate transcription factors such as NF-KB and AP-1 through the recruitment of various adaptor proteins and kinases such as TRADD, RIP, NIK and TRAF. NFKB is able to initiate the up-regulation of inflammatory responses (Ashkenazi and Dixit, 1998; Wallach et al., 1998), even promoting proliferation of the cell (Tartaglia et al., 1993). FLIP molecules are a group of negative regulators of death receptor-induced apoptosis that resemble caspase-8 but lack its enzymatic activity. FLIP is able to bind to FADD thereby subverting the processing of Caspase 8 which in turn interferes with the death cascade (Yeh et al., 2000).

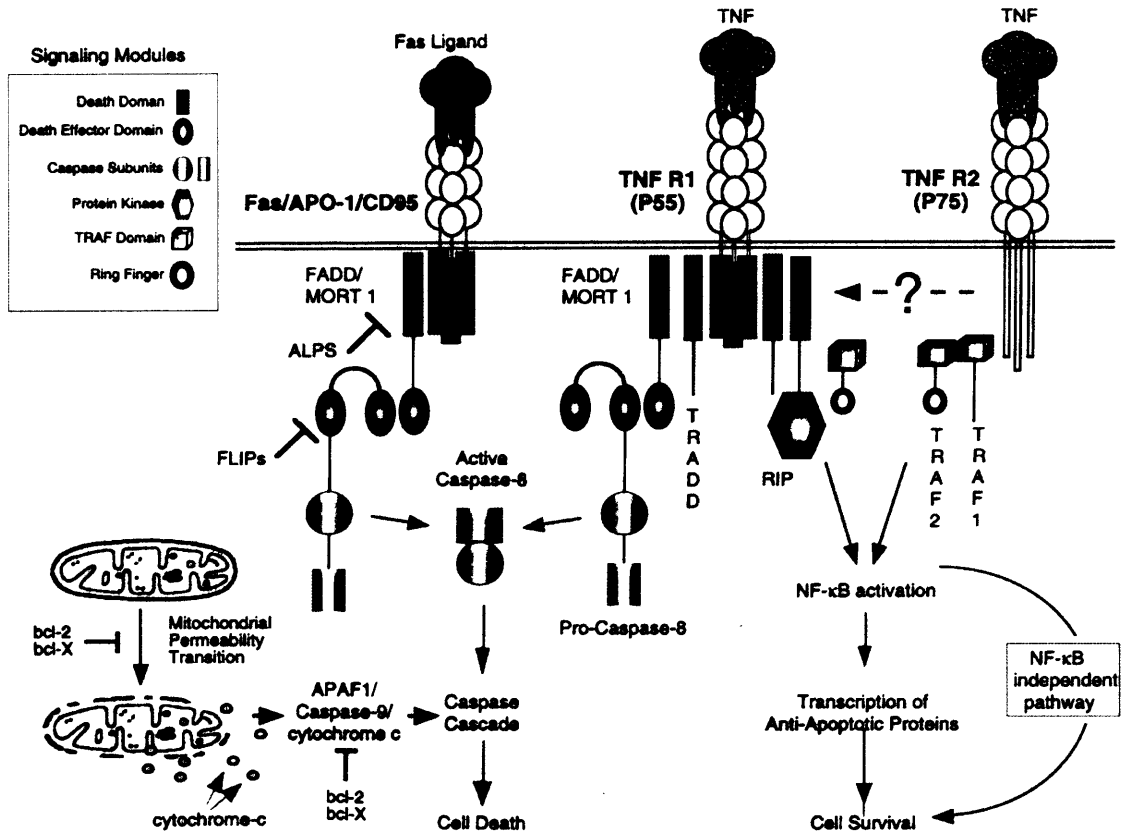

Figure 1.13: Signaling by Fas/APO-1/CD95, TNFR1 and TNFR2.

For active apoptosis, trimeric death cytokines, FasL, and TNF interact with trimers formed by individual receptor molecules. This interaction promotes the recruitment of downstream signaling molecules distinct for each receptor. Fas induces apoptosis via recruitment of FADD and caspase-8. Shown are the signaling impediments caused by the ALPS mutations in the death domain and by FLIPS in FADD: caspase-8 DED interactions. TNFR1 can signal either for apoptosis through TRADD and FADD, or deliver anti-apoptotic signals via recruitment of alternate signaling complexes consisting of TRADD, RIP, and TRAF-2. The anti-apoptotic signaling complex probably activates the NF-κB pathway through the NIK kinase that binds TRAF-2. NF-κB presumably induces the expression of as yet unidentified survival molecules. Signaling through TNFR2 proceed through the recruitment of TRAF proteins, most

likely a complex of TRAF1/2 as indicated, although other TRAF proteins can bind TNFR2 *in vitro*. The TRAF1/2 complex can also activate NF-κB, but experiments in TRAF-2-deficient mice have revealed other NF-κB-independent anti-apoptotic pathways. Paradoxically, TNFR2 signaling may also sensitize cells for apoptosis through TNFR1 (dashed line), although the mechanism is not well understood. The cytoplasmic mechanism of caspase activation is apparently initiated by a mitochondrial permeability transition that allows the release of cytochrome c. Cytochrome c causes the association of APAF1 and caspase 9, leading to the activation of caspase 9 and triggering a caspase cascade. Bcl-2 family proteins can block apoptosis at the point of mitochondrial permeability transition and possibly by directly inhibiting the APAF-1/Caspase-9 complex, which activates downstream caspases.

1.11.9.2 Intrinsic apoptotic pathway

The intrinsic death pathway is influenced by growth factor deprivation, DNA damage or TCR triggering (Krammer et al., 2007; Strasser et al., 1995) and is governed by members of the Bcl-2 family, which can be divided into two groups: the negative and the positive regulators.

At least 24 proteins, encoded by 20 genes (Adams and Cory, 1998), function by sensing cellular stress via signals from various sources such as the endoplasmic reticulum, the cytoskeleton, the nucleus and the mitochondria.

Anti-apoptotic members include Bcl-2, Bcl-XL, Bcl-w, Boo, A1, Mcl1 and BclB (Boise et al., 1993; Gibson et al., 1996; Ke et al., 2001; Kozopas et al., 1993; Lin et al., 1996; Song et al., 1999; Tsujimoto et al., 1985). The anti-apoptotic members of the family all share 3 or 4 homology domains called BH (Bcl2-homology) regions. They localize to the mitochondrial outer membrane (Nguyen et al., 1993) as well as the cytoplasmic faces of the endoplasmic reticulum (Krajewski et al., 1993) and nuclear envelope (Lithgow et al., 1994).

The pro-apoptotic members include Bax, Bcl-Xs, Bak, Bok, Bad, Bik, Bid, DP5, Blk, Noxa, Puma, Bcl-G, Bmf and Bim (Oltvai et al., 1993),(Boise et al., 1993), (Kiefer et al., 1995),(Hsu et al., 1997),(Yang et al., 1995),(Boyd et al., 1995),(Wang et al., 1996),(Imaizumi et al., 1997),(Hegde et al., 1998),(Oda et al., 2000),(Nakano and Vousden, 2001), (Guo et al., 2001), (Puthalakath et al., 2001),(O'Connor et al., 1998).

These proteins can be divided into two groups based on the number of BH domains that they contain. Bax, Bok, Bak, Bcl-Gl and Bcl-Xs contain multiple BH domains whereas Bik, Blk, Hrk, Bad, Bid, Bcl-G, puma, noxa, bmf and Bim contain a short BH domains and are therefore refereed to as BH3-only proteins (Huang and Strasser, 2000).

These proteins induce apoptosis by binding and antagonizing pro-survival members (Kelekar and Thompson, 1998). Cell is death normally avoided by sequestering these proteins away from the prosurvival members; key triggers facilitate their release (Puthalakath et al., 1999; Puthalakath et al., 2001) thereby allowing them to interfere with the apoptosis repressors.

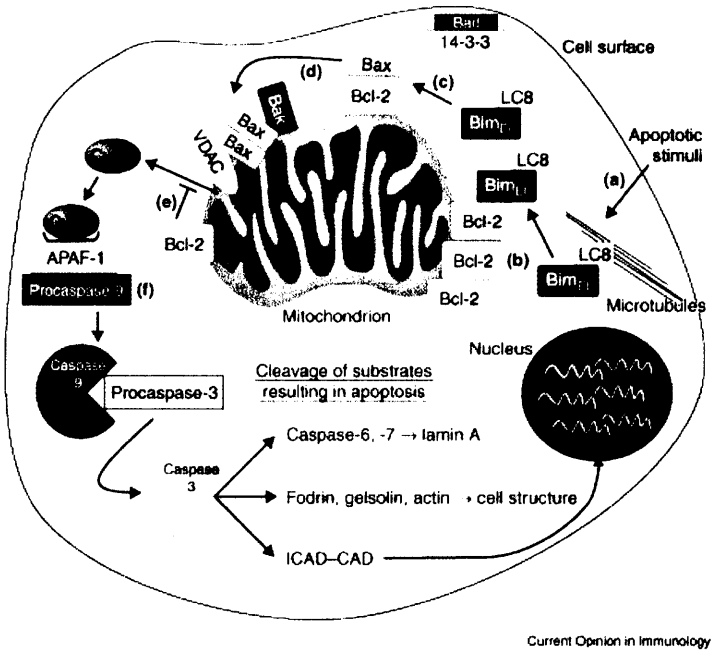

Figure 1.14: Illustration of apoptosis driven by Bcl-2 family members in activated T cells.

Pro-apoptotic BH3-only Bcl-2 family members Bad and Bim (BIMEL signifies 'extra long' Bim) are sequestered in the cytosol by 14-3-3 and light chain 8 (LC8) of the dynein motor complex. (a) Apoptotic stimuli (i.e. activation of T cells by antigen in vivo) by an unknown mechanism signal Bim to release from microtubules. (b) High levels of Bcl-2 may prevent death because they sequester Bim. High levels of Bim by an unknown mechanism signal (c) Bax and/or (d) Bak to form pores in mitochondria and, in association with VDAC, cause the release of cytochrome C (Cyt C) from mitochondria. (e) Bcl-2 has also been shown to block Cyt C release from mitochondria. (f) Once released, Cyt C interacts with APAF-1 and procaspase-9. This complex favors the activation of caspase-9 which, when activated, cleaves and activates caspase-3 which in turn cleaves other caspases, the inhibitor of caspase-activated DNAse (ICAD) and molecules important in cell integrity.

The Bcl-2-interacting mediator, Bim, is particularly relevant to chronic viral infection because it is capable of down-regulating antigen-specific CD8+ T cell responses (Bouillet et al., 1999; Grayson et al., 2006; Hildeman et al., 2002; O'Connor et al., 1998; Pellegrini et al., 2003).

Both cytokine deprivation as well as TCR triggering contributes to increases in Bim expression (figure 1.15), however the latter mechanism appears to be particularly important given the context of repeated antigenic stimulation during persistent infection.

The upstream pathway is not well understood, however, downsteam events involve the adaptor protein Apaf-1 that is required to cluster Caspase-9 in order to enhance its autocatalytic activity which in turn is necessary for apoptosis. Cytoplasmic Apaf-1 is activated following engagement with cytochrome c which is normally sequestered in the mitochondria; the accessory proapoptotic mediators Bax and Bak mediate the release of cytochrome c but are normally maintained in an inactive state by Bcl-2 and its homologues (Cheng et al., 2001; Zong et al., 2001).

However, the binding of Bim to Bcl-2 initiates Bax and Bak functionality (Willis et al., 2007). The difference between cell survival and cell death is governed by the equilibrium between the pro and anti-apoptotic Bcl-2 family members. IL-7 can upregulate the Bcl-2 family members (Akashi et al., 1997; Maraskovsky et al., 1997) whereas the proapoptotic proteins can be increased by alternative extracellular ligand signalling (Puthalakath et al., 2001; Zha et al., 1996).

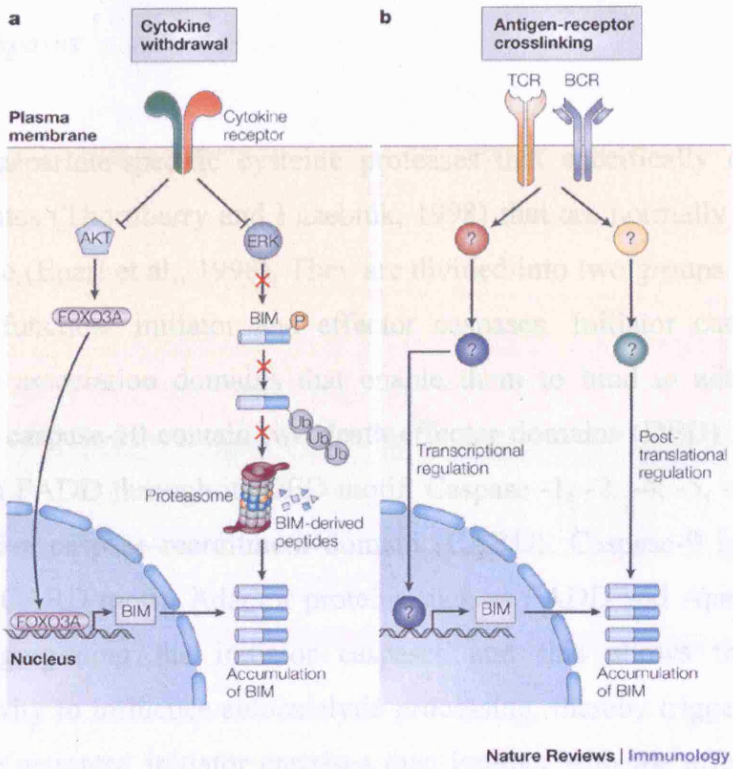

Figure 1.15: Mechanisms for BIM activation in lymphoid cells.

(a) Cytokine deprivation can activate the pro-apoptotic activity of Bim (B-cell lymphoma 2 (BcL-2)-interacting mediator of cell death) in lymphoid cells through at least two pathways. Loss of cytokine-receptor stimulation causes inactivation of the kinase AKT, which in turn leads to activation of the transcription factor FOXO3A (forkhead box O3A), resulting in increased BIM mRNA synthesis. Cytokine deprivation also leads to inactivation of the kinase ERK (extracellular signal-regulated kinase), causing loss of BIM phosphorylation, which targets BIM for ubiquitylation and proteasomal degradation.

(b) During apoptosis induced by antigen-receptor crosslinking, increased levels of BIM mRNA and BIM protein have been observed, but little is known about the signalling pathways that are responsible for this. BCR, B-cell receptor; TCR, T-cell receptor.

Adapted with permission from Nature Publishing Group. Nature Reviews Immunology; Strasser, A; 2005: 5(3)

1.11.10 Caspases

Caspases are aspartate-specific cysteine proteases that specifically cleave critical cellular substrates (Thornberry and Lazebnik, 1998) that are normally maintained in an inactive state (Enari et al., 1998). They are divided into two groups based on their structure and function: initiator and effector caspases. Initiator caspases contain protein-protein association domains that enable them to bind to adaptor proteins. Caspase 8 and caspase-10 contain two death effector domains (DED) allowing them to interact with FADD through its DED motif. Caspase -1, -2, -4, -5, -9, -11 and -12 contain instead a caspase recruitment domain (CARD). Caspase-9 is able to bind Apaf-1 via its CARD motif. Adaptor proteins such as FADD and Apaf-1 exert their effects by aggregrating the initiator caspases and this allows their low-level enzymatic activity to influence autocatalytic processing, thereby triggering the death cascade. These activated initiator caspases then interact with the effector caspases. Subsequent proteolytic clevage of cellular proteins contributes to destabilizing the integrity of the cell. Ultimately, a caspase activated DNAse (CAD) digests the genomic DNA.

1.11.11 Cross-presentation of antigen to CD8+ T cells

The majority of naive and resting T cells are located in secondary lymphoid tissue (SLT) that includes the spleen, lymph nodes and peyers patches (figure 1.16). These cells continually recirculate between different parts of these organs in a cycle that takes approximately one day to complete. This trafficking ensures that each individual compartment of the secondary lymphoid tissue has an optimally diverse TCR repertoire. Professional antigen presenting cells (pAPCs) stationed throughout the body transport antigen from peripheral tissues to lymph nodes where they are presented to the residing population of T cells.

CD8+ T cell priming requires presentation of antigen by MHCI complexes (Bevan, 1987). This normally occurs in all infected cells and serves to identify intracellular pathogens to facilitate their destruction and limit replication. However, certain (uninfected) APCs are capable of taking-up exogenous antigen and presenting it on MHCI complexes on the cell surface (Pozzi et al., 2005). This is particularly important as it prevents viruses escaping immune recognition through the downregulation of MHC expression (Ahn et al., 1996a; Fruh et al., 1995; Levitskaya et al., 1997; Ploegh, 1998; Tortorella et al., 2000). One of the earliest observations of cross-presentation in humans was made during an investigation of human T cell lines specific for HBsAg (Jin et al., 1988).

Subsequent studies have shown that soluble antigen that enters APCs by fluid-phase endocytosis is cross-presented less efficiently compared to particulate matter unless present at high concentrations (Carbone and Bevan, 1990; Falo et al., 1995; Rock et al., 1990). Particulate antigens >1μm in size are taken up by phagocytosis (Allen and Aderem, 1996), whereas smaller particles and soluble antigen are taken up by macropinocytosis.

Figure 1.16: Schematic illustrating the location and distribution of lymphoid organs in the body.

(http://www.nevdgp.org.au/info/ccv/patients/images/lymphatic.gif)

The mechanism of cross-presentation is thought to involve fusion of phagosomes with endoplasmic reticulum-derived vesicles bringing together the antigen, MHCI complexes and the components required for loading (Ackerman et al., 2003; Cresswell et al., 1999; Guermonprez et al., 2003; Houde et al., 2003) (figure 1.17). The antigens are then transported to the cytosol adjacent to the phagosome and are degraded by proteasomes into peptides that are transported back to the phagosome by the TAP complex for loading (Jarosch et al., 2002; Matlack et al., 1998; Romisch, 1999; Wiertz et al., 1996b). Finally, the fusion of phagosomes with the surface membrane exposes the peptide loaded MHCI. Alternatively, a TAP/proteasome-independent vacuolar pathway can also contribute to antigen cross-presentation but has not been fully characterized (Bachmann et al., 1995; Rock, 1996; Shen et al., 2004). Other, studies have demonstrated that cross-presentation of soluble proteins from HBV, HCV- and HIV-1 can be enhanced by reducing endocytic acidification by cholorquine or ammonium chloride treatment (Accapezzato et al., 2005).

Although B cells (Ke and Kapp, 1996), endothelial cells (liver sinusoidal endothelial cells) (Limmer et al., 2000) and macrophages (Debrick et al., 1991) are all capable of cross-presenting antigens that include soluble proteins, immune complexes, intracellular bacteria, parasites and cellular antigens (Kurts et al., 1996), (den Haan and Bevan, 2002), (Pfeifer et al., 1993), (Belkaid et al., 2002), (Albert et al., 1998). CD8+ DCs are the main subset that can effectively cross-present antigens to CD8+ T cells (Iyoda et al., 2002; Valdez et al., 2002).

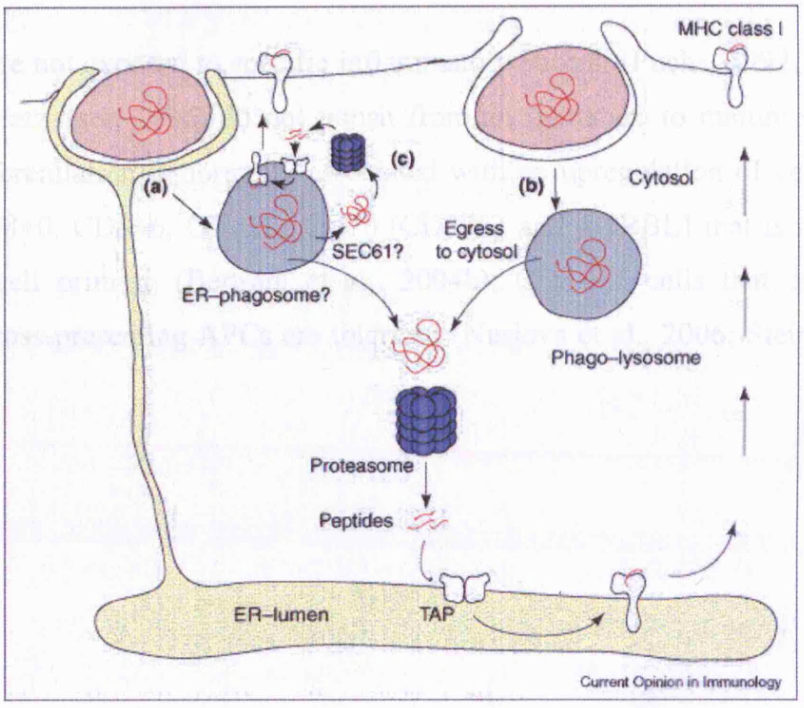

Figure 1.17: Major histocompatibility complex (MHC) class I presentation pathways.

Possible routes that phagocytosed antigens take to reach proteasomes in the cytosol. In (a), ER-derived phagosomes were proposed to shelter phagocytosed antigens and to transport them to the cytosol via the Sec61 channel. The pathway outlined in (b) represents the conventional route of phagocytosis, whereby the plasma membrane engulfs the incoming particles to create a nascent phagosome, which then fuses with elements of the lysososmal system. From there antigens would access the cytosol. According to (c), ER-derived phagosomes contain the necessary components for cross-presentation, mediating retrotranslocation via Sec61 to access proteasomes on the cytosolic surface of the phagosome, resulting in peptides further re-imported via TAP into the phagosome for loading onto MHC-I molecules, followed by transport to the plasma membrane.

1.11.12 Cross-tolerance

APCs that are not exposed to specific inflammatory stimuli (Fuchs, 1992; Janeway et al., 1989; Matzinger, 1994) do not transit from an immature to mature status. This cellular differentiation is normally associated with an upregulation of costimulatory ligands [CD8+0, CD8+6, OX40L, CD70 (CD27L) and 4-1BBL] that is required for optimal T cell priming (Bertram et al., 2004b); CD8+ T cells that engage with immature cross-presenting APCs are tolerised (Nurieva et al., 2006; Steinman et al., 2003b).

1.12 Gene expression analysis

Cellular responses are the result of complex intracellular events that involve multiple players acting either in parallel or in series. The cellular genome itself contains the information that is necessary to initiate such a response. In accordance with this central dogma, information travels from genomic DNA to RNA and finally to protein; the cellular genome is transcribed into messenger RNA (mRNA) and this is then translated by ribosomes into polypeptides, the majority of which require subsequent modification to confer functional activity.

The most common approach towards understanding specific cellular function (and dysfunction) follows a reductionist method based on the identification of a single gene or its protein product followed by attempts to relate it to the phenotype under investigation. Traditional methods uses in these studies have been northern blots, RNase protection assays, western blots and more recently flow cytometry. Studies utilising this approach have contributed significantly to our understanding of specific cellular processes, however they are limited in scope, particularly when complex serial and/or parallel multi-gene events are involved.

Changes in protein content are subsequent to mRNA transcription, therefore cellular levels of mRNA are to some extent representative of protein content. Specifically, an increase or decrease in the expression of a particular mRNA species is indicative of an increase or decrease in the expression of the corresponding protein respectively. High throughput gene expression analysis is therefore a highly effective tool to assess cellular function by simultaneously measuring the quantities of multiple gene transcripts.

1.13 DNA Microarrays

Several methodologies have been used for the analysis of gene expression. These include serial analysis of gene expression (SAGE), cDNA library sequencing, differential display, cDNA subtraction, QPCR, and gene expression microarrays. The particular advantage of microarray analysis for comparative studies on biological samples is the capacity to simultaneously examine the gene profile of a very large number of genes from a relatively small sample. Microarrays measure the hybridization between DNA that has been immobilized to a rigid platform (glass, nylon, plastic) to some form of mRNA representation from the sample under study. The DNA is arranged in concise spots in an ordered matrix pattern and allows a separate but simultaneous analysis to occur for each distinct spot. This makes it possible not only to measure the quantity of a specific gene transcript but also to assess the relationship between different transcriptional specificities. Gene expression microarray technology is mainly conducted using oligonucleotide or cDNA arrays, differing in the manner in which the DNA sequences are put onto the array as well as the length of the sequences used.

1.13.1 Oligoarrays

This approach to microarrays has been popularized by Affymetrix with their range of GeneChips, that involve the use of oligonucleotides that are covalently linked to glass slides. These arrays utilize a comparison of the hybridization of the sample mRNA (which has been labelled with a specific marker - biotin) to a pair of probes for each gene that are composed of 11 to 20 pairs of oligonucleotides of 25 bases in length. One probe sequence is a perfect match [also referred to as the perfect match (PM)] and is obtained from the gene sequence. The second probe is an imperfect match [known as the mismatch (MM)] and is constructed by purposely altering the middle (13th) base of the PM to alter the efficiency of binding. The purpose of MMs is to control for experimental variation and non-specific hybridization in the sample. Ultimately, oligonucleotide arrays measure the fluorescence intensity of one reporter

dye (streptavidin – PE) and obtain two sets of readings; one for the PM and the other for the MM, from which the expression of specific genes is evaluated through accessory computational algorithms.

1.13.2 cDNA arrays

In DNA arrays, mRNA from a biological sample is converted to cDNA by reverse transcription using dNTPs that are labelled with a fluorescent dye. Although basic modifications of the labelling process do exist they ultimately result in a fluorescently tagged anti-mRNA sequence. This product is then hybridized to DNA sequences (which can be cDNA generated from PCR products or oligonucleotide sequences) that have been immobilized to a rigid platform such as a glass slide, with unique DNA sequences spotted in discrete circular areas or spots.

Following hybridization and removal of unhybridized sample, a laser scanner is used to measure the fluorescence intensity and stores data at the level of individual pixels. Higher fluorescence corresponds to higher hybridization; this in turn corresponds to the quantity of the transcript in the sample tested. Computational image analysis algorithms are then used to calculate the fluorescence intensity of individual spots as well as defined areas of background fluorescence for each spot.

1.13.2.1 Comparative analysis through dual fluorescence

An extension of the basic principal of DNA microarrays involves the use of two different fluorescent dyes and is known as dual-colour microarrays. Here, mRNA from the sample, referred to as the test RNA, is reverse transcribed into cDNA in a reaction that incorporates a fluorescently labelled dye (Cy5; red). A second source of mRNA is also reversed in the same way but with another fluorescent colour (Cy3; green); this is referred to as the reference RNA. Equivalent amounts of the labelled cDNAs are then mixed together and applied onto one array where they competitively

hybridise to their respective probes. Thus, if a specific transcript is highly expressed in the test sample relative to the reference, it will appear more red than green when the array is scanned. This quality can be measured precisely and results in two values corresponding to the fluorescence intensity for Cy5 and for Cy3 for each gene spot on an array (figure 1.19).

The use of a unique stock of reference RNA on multiple arrays each with a different test RNA sample creates the possibility of cross comparing gene expression data of different arrays by comparing the ratio of Cy5 to Cy3 for any given gene.

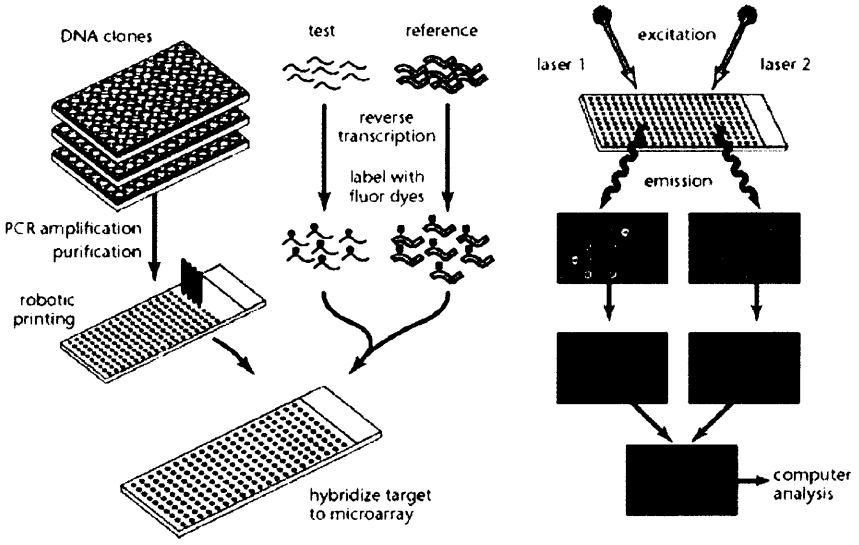

Figure 1.18: cDNA microarray schema.

Templates for genes of interest are obtained and amplified by PCR. Following purification and quality control, aliquots are printed on coated glass microscope slides using a computer–controlled, high–speed robot. Total RNA from both the test and reference sample is fluorescently labelled with either Cy3– or Cy5–dCTP using a single round of reverse transcription. The fluorescent targets are pooled and allowed to hybridize under stringent conditions to the clones on the array. Laser excitation of the incorporated targets yields an emission with a characteristic spectra, which is measured using a scanning confocal laser microscope. Monochrome images from the scanner are imported into software in which the images are pseudo–coloured and merged. Information about the clones, including gene name, clone identifier, intensity values, intensity ratios, normalization constant and confidence intervals is attached to each target. Data from a single hybridization experiment is viewed as a normalized ratio (that is, Cy3/Cy5) in which significant deviations from 1 (no change) are indicative of increased (>1) or decreased (<1) levels of gene expression relative to the reference sample. In addition, data from multiple experiments can be examined using any number of data mining tools.

Printed with permission from Nature Publishing Group. Nature Genetics; Duggan, DJ; 1999: 21.

1.13.2.2 Experimental variability and counter strategies to minimize it

A major advantage of using two-colour arrays is the possibility to overcome cDNA spot printing variations that can occur during the manufacturing stage. If spotting is not consistent, certain arrays may have significantly different sized spots for any given gene and therefore the interpretation of single-colour hybridizations would not accurately represent true gene expression. As two-colour arrays are based on a competitive hybridization, variations in spot size do not matter; Cy5 to Cy3 ratios would be unaffected.

Variability in data measurements can however occur at multiple levels and can obscure the essential biological signals that are being sought. Such error arises at various stages of array preparation and processing and must be acknowledged and vigorously controlled where possible to minimize overall error. For example, manufacturing error can be traced to amplification of the gene sequence, purification of the product, concentration of DNA used for spotting, efficiency of immobilization of DNA to the array and morphology of the spot. Sample preparation may suffer variations in RNA extraction and amplification procedures and differential labelling efficiencies. Dye specific variations include standard rates of decay, susceptibility to environmental factors such as ozone, and differential incorporation efficiencies due to physical size. Hybridization can be subject to variability as a result of cross-hybridization due to similarity of probe sequences, changes in temperature and humidity, environmental contaminants, and efficiency of the subsequent washing procedure to remove non-hybridized sample. Lastly, variable background fluorescence in the scanned arrays can interfere with the true spot intensity by decreasing the signal to noise ratio. High scanning intensities improves signal quality but concomitantly increases the risk of signal saturation that occurs when certain spots fluoresce above maximum intensities thresholds of the scanner.

Ultimately, the most favoured strategy for dealing with uncertainties at the individual spot level is to mark the spot under question for exclusion in the final analysis. As for

the spots that pass this screening, although the extent of the individual errors may be minimal, the cumulative effects as described above can translate to significant deviations in the final data and thus must be complemented with appropriate controls.

Replication in arrayed samples strengthens the reliability of the conclusions. This can occur in two forms. One form, biological replicates, involves the analysis of samples from different sources and is critical when genome biometry is large. A second form, technical replicates, involves multiplications of the same sample that establish the fidelity of the processes from initial crude sample (cell lysate), past RNA extraction and right through to the scanning of the processed array. To complement this, internal controls on each array, such as housekeeping genes and negative controls, help assess the source of experimental error.

1.13.2.3 Primary data output

Processed array slides are normally scanned at a resolution of 5 or 10μm; excitation and fluorescence is systematically measured over an area of 5 μm x 5 μm or 10μm x 10mm that correspond to pixels of 25μm^2 or 100mm^2 respectively. After acquisition, an algorithm is used to define the location and perimeter of each spot and a local background. Computational tools can then be applied to segment and summarize the data (mean and median intensity value with standard deviation for Cy5 and Cy3) contained in the user-defined zones encompassing each spot. As mentioned previously, algorithms also cater to the extraction of local background fluorescence for every spot and can subsequently be subtracted from specific spot readings. However, if low intensity readings are generally observed, this step can be by-passed, while still allowing conservative interpretations of differential gene expression. Finally, the quality of the array data can be controlled by visual inspection of scatterplots of differential expression against overall intensity of the individual channels on an array (Cy5 versus Cy3) commonly referred to as the MA plot. These plots can be used to identify intensity-related biases and variances, saturation effects

and other artefacts. For example, significant fish-tails at low intensities indicate that spot-level background subtraction cannot be reliably executed.

1.13.2.4 Data processing

Handling data of thousands of genes of multiple samples requires appropriate tools that are able to cope with the volume and complexity of such information. The data are normally held and manipulated in microsoft excel into a format that includes three main parameters: the gene name, the gene identification number (linked to a unigene or genebank accession number - where current detailed information of the individual gene can be found) and the absolute or relative expression value.

1.13.2.5 Log transformation

Spot intensity values are usually converted to log values and/or log ratios of intensities in order to force the data to approach a normal distribution. This also facilitates the interpretation of gene expression changes. The use of a common reference gene in all the arrays provides a gene specific constant across all the arrays. Log_2 transformation is preferred to Log_{10} as it allows an easier interpretation of the data. In this way, a Log_2(Cy5/Cy3) of 1, 0 and -1 correspond to a two-fold up-regulation, no change, and a two-fold down-regulation respectively.

1.13.2.6 Normalization

In order to compare the gene expression profiles of data across multiple arrays the random and systematic variation that are introduced during array manufacture, sample preparation, and array processing have to be minimized through a process called normalization.

The basis of array normalization rests on the fact that log transformation forces the data into a normal distribution pattern. Global normalization assumes that the Cy5 and Cy3 intensities are related by a constant factor, and the expression ratio of the median gene on the population is zero based on the observation that the majority of genes do not alter their expression. Thus, whole data sets can be shifted so that they centre about a median of 0 while still conserving the standard deviation (which is a measure of the expression ratio data spread) within the dataset.

Despite normalization, within-group variation of individual gene expression can be significantly variable due to the heterogeneity of the source sample, and this requires statistical tools that are able to weight the differences accordingly. For example, within a group, replicates of one sample can be tagged so that the data analysis tools can consider this when assessing relationships to other individual samples in the group.

1.14 Data analysis

When it comes to the selection of differentially expressed genes, several approaches can be used to evaluate the degree of reliability of the data. The use of *p*-values has been criticised due to the multiplicity of the comparisons involved. A second method relies on calculating the probability of differential regulation. The third and currently most popular approach is to use the false discovery rate (FDR) that is an estimate of the fraction of genes that are truly altered among a selection of genes that are regarded as significant (Benjamini and Hochberg, 1995).

1.14.1 Significance Analysis of Microarrays

Significance Analysis of Microarrays (SAM) is an analytical tool that was developed by Tusher et al. (Tusher et al., 2001). Its main function is to correlate gene expression differentials between two groups with an outcome parameter by thresholding an appropriate test statistic and reporting the *q* value and FDR of each test based on a set

of sample permutations. The statistical demands of identifying differentially expressed genes requires multiple hypothesis testing where multiple hypothesis tests are conducted simultaneously on all the genes under study. In statistical hypothesis testing, a null hypothesis is tested against an alternative hypothesis to determine the outcome. The null hypothesis is rejected in favour of the alternative if there is sufficient evidence to support the latter. This involves the rejection of the null hypothesis if its corresponding statistic lies in a predetermined rejection range. Additionally, hypothesis testing also involves an evaluation of the probability of rejecting the null hypothesis when it is actually valid (known as a false positive) as well as calculating the probability of rejecting the null hypothesis when the alternative hypothesis is actually true (termed power).

Differential gene expression involves the execution of four steps. First, a statistic must be generated for each gene. Then, the corresponding null distribution is calculated, this is followed by the specification of the rejection region, and finally the number of false positives must in some way be assessed or controlled. When determining the statistical significance of the difference in gene expression between two groups, the null hypothesis for each gene is that the observed data has a common distributional parameter within the groups. This can be the mean expression level. A statistic that is a function of the data is formed for each gene (t) and a significance region is defined for this statistic. If the statistic falls in this region the gene is noted as exhibiting differential gene expression.

Using this approach, two forms of errors can occur. Type 1 errors (false positives) occur when the statistic is significant but the gene is not expressed at a significant differential. Type 2 errors (false negatives) occur when the statistic is not significant but the gene is in fact differentially expressed. In multiple hypotheses testing each gene may includes both type 1 and type 2 errors and the measurement of the overall error rate becomes unclear. To overcome this complication, one can define a measure of compound error, such as the FDR (Benjamini and Hochberg, 1995).

In the SAM PLOT (see chapter 4; figure 4.14), the genes conforming to the null hypothesis lie along the 45° diagonal line. The dotted tram lines above and below this are set according to the user defined FDR. All genes to the right of the tramline intersection, regardless of whether they are above or below this line are considered significant. The same is true for genes below and to the left of the lower tramline.

Microarray studies involve a large number of individual tests (genes) and are very much an exploratory analysis, with the aim of identifying a group of differentially expressed genes of which a small proportion are expected to be false positive. Ultimately, the aim is to shortlist a subset of genes from the totality of the test data that can then be focused on for a more detailed analysis. The power of SAM lies in the ability to define the rejection regions to accommodate the proportion and degree to which certain genes are over/under expressed. Additionally, a multiple hypothesis testing error rate takes into consideration multiple comparisons while simultaneously limiting the proportion of false positives that occur within the genes selected as significant.

1.14.2 Clustering

Clustering algorithms can be used to arrange a number of objects (such as genes or samples) into groups where the expression of genes within groups is more similar compared to the whole group.

1.14.2.1 Non-hierarchical clustering

Non-hierarchical clustering locates a single partition without nesting and is more suited to identifying candidate subgroups within complex data. K-means clustering and self organising maps (SOMs) are the most common methodologies. In K-means clustering an algorithm randomly assigns an object to one of several K clusters, the number of which have to initially be defined by the user (Hartigan and Wong, 1979).

Distances between the clusters are then calculated based on the mean vector within each cluster. An iterative process is then used to maintain or relocate objects to other clusters to increase trans-cluster variance and decrease within-cluster variance. The process is terminated when no further improvements can be made. SOMs function by reducing dimensionality by generating a map that outlines the similarity among object, with similar objects groups together.

1.14.2.2 Hierarchical clustering

Hierarchical clustering on the other hand generates a series of successively nested clusters that represent the distances between the expression profiles in a tree or dendogram, where the data are made up of a series of partitions that extend from a single cluster down to sub clusters and terminate in a single gene or sample. This methodology is highly effective at facilitating visual interpretation. Clustering can be agglomerative (bottom-up), where every object starts by belonging to its own cluster and is then matched to the most similar other object. This process continues until there is only one cluster containing all the objects. Alternatively, clustering can be divisive, which is the opposite process and starts with all the objects and divides groups according to similarity until single objects remain. Each method usually produces unique hierarchies. Gene expression analysis are more suited to divisive analysis because these clusters are more stable, as the focus is on large clusters and fewer permutations are required to generate the clusters (compared to agglomerative clustering). Finally, unsupervised clustering can be used to screen data in an explorative manner for patterns of differential gene expression, which can then be correlated to the composition of objects within the group.

2 *Chapter 2: Material and Methods*

2.1 Patients and Volunteers

Patients (table 2.1) were recruited from the Mortimer market centre, London, with full ethics approval and informed consent. HBeAg and anti-HBe antibody status was tested with the commercial enzyme immunoassay; Murex Diagnostics. HBV DNA viral load was quantified by the Roche Amplicor Monitor Assay; Roche Laboratories. All patients were negative for antibody to HIV-1 and HIV-2; Ortho Diagnostics. None of the patients recruited had received antiviral or immunosuppressive therapy.

2.2 Separation of peripheral blood mononuclear cells from whole blood

Venous blood was drawn into EDTA tubes and processed on the same day. Blood was layered directly onto ficoll at a ratio of 2:1 (blood:ficoll) and spun at room temperature at 1600rpm; 24 minutes (brake off). Plasma was removed up to 5mm above the interface with the ficoll and the PBMC layer was collected into a 20ml universal predispensed with 10mls of RPMI. Following two cold washes in RPMI (first spin at 1800rpm and the second at 1600), the cell pellet was resuspended in complete aMEM (10% FCS); ~1ml for each 10mls of blood processed. Cell samples were normally placed on ice until required for assays/freezing.

2.3 Cell counting

Cells were counted with a haemocytometer (improved neubauer) under a light microscope; 10µl tryphan blue + 10µl of a 1/10 dilution of the stock (10µl cells + 90µl RPMI); equivalent to a dilution factor of 20; the dilution factor was altered if necessary. Formula for cell yield: Number of cells in the central grid x dilution factor x 104 = [cells/ml].

Table 2.1: Patient details.

Patient	age	sex	viral load (IU/ml)	ALT	serology	cDNA arrayed (+ purified*)	Bim	Mcl-1	Rescue (VAD)	
							10 day	10 day	ex vivo	10 day
Resolved										
R1	NA	F	ND	NA	HBsAg-	Y*	Y	Y	N	Y
R2	NA	M	ND	<50	HBsAg-	N	Y	Y	N	Y
R3	50	M	ND	NA	HBsAg-	N	N	N	N	Y
R4	41	NA	ND	NA	HBsAg-	Y	Y	Y	N	Y
R5	32	M	ND	10	HBsAg-	N	Y	Y	N	Y
R6	38	M	ND	<50	HBsAg-	Y	Y	Y	N	Y
R7	26	M	ND	32	HBsAg-	N	Y	Y	N	N
R8	35	M	ND	<50	HBsAg-	N	Y	Y	N	N
R9	39	M	ND	74	HBsAg-	N	Y	Y	N	N
R10	NA	F	ND	NA	HBsAg-	Y*	N	N	N	N
R11	NA	M	ND	NA	HBsAg-	Y	N	N	N	N
Chronic										
C1	25	M	0.42E	270	HBeAg+	N	Y	Y	N	Y
C2	49	F	1.1E	151	HBeAg-	N	Y	Y	N	Y
C3	31	M	180	92	HBeAg-	N	Y	Y	N	N
C4	29	F	1,100	NA	HBeAg-	N	Y	N	N	N
C5	38	M	510E	206	HBeAg+	N	Y	Y	N	Y
C6	28	F	7.9E	261	HBeAg+	N	Y	Y	N	Y
C7	50	F	0.22E	63	HBeAg-	N	Y	Y	N	Y
C8	NA	M	>10E	<50	HBeAg+	Y*	N	N	Y	Y
C9	NA	M	570	<50	HBeAg-	N	N	N	Y	Y
C10	44	M	0.065E	49	HBeAg-	N	N	N	N	Y
C11	31	M	4,500	NA	HBeAg-	N	Y	Y	Y	Y
C12	35	M	220E	42	HBeAg+	N	Y	Y	N	Y
C13	31	M	>10E	<50	HBeAg+	Y	N	N	N	Y
C14	42	F	NA	17	HBeAg-	N	Y	Y	Y	N
C15	45	M	110	32	HBeAg-	N	Y	Y	Y	N
C16	71	M	2,900	22	HBeAg-	N	Y	Y	Y	N
C17	30	F	770	31	HBeAg-	N	Y	Y	N	N
C18	33	F	BLQ	17	HBeAg-	N	Y	Y	N	N
C19	NA	M	>10E	NA	HBeAg+	Y	N	N	N	N

NA=data not available; E = x 1,000,000

2.4 Cell storage

Working quickly, PBMCs were pelleted (1600rpm; 10mins), supernatant decanted, resuspended at 10million/ml in FCS (10% DMSO), aliquoted into cryovials (normally 5million/vial), placed in a Mr. Frosty freezing chamber (Fisher Scientific, UK) and put into a –70°C freezer for 24 hours. The vials were then transferred to a liquid nitrogen tank for long-term storage.

2.5 Cell retrieval from liquid nitrogen storage

Working quickly, cells were semi-thawed in a water bath (37°C) and dispended into 20ml of RPMI, mixed well, spun (1600rpm; 10mins), resuspended in complete aMEM + 10% FCS; approximately 500µl for every 5million vial and counted [dilution factor (with tryphan blue) 1:1].

2.6 Cell Culture (HBV-specific CD8+ T cell enrichment)

100µl of cMEM (10% FCS & 40U/ml IL-2) was dispensed into the appropriate number of wells in a 96 well round bottom plate. Appropriate peptides (Chiron Mimotopes; >90% pure by HPLC analysis) were added where required (2µM). 100µl of cell sample (diluted to 3 x 106/ml) was then dispensed into each well and incubated at 37°C; 4% CO_2; for 10 days. IL-2 (Roche) was supplemented on day 4; 100µl of medium was aspirated from the top of each sample and replaced with complete aMEM with 40U/ml IL-2. The frequencies of peptide specific cells were evaluated by tetramer staining or by intracellular cytokine staining (ICS) for IFN-γ on day 10.

2.7 Multimer staining

For tetramer/pentamer staining, total PBMCs were stained with the manufacturers recommended quantity of multimer at 37°C, washed (PBS + 1%FBS) and then

stained with anti-CD8 (BD Biosciences); 15mins in the fridge. The cells were then pelleted, staining medium removed, washed once in PBS (Sigma-Aldrich, St. Louis, MO) (1% formaldehyde (Anachem) & 1% FCS), resuspended in the same solution.

2.8 Intracellular cytokine staining

For the ICS, 100µl medium was aspirated from the top of the wells, cells were resuspended in the remaining 100µl and then restimulated with peptide (2µM) for 5 hours; multiple wells that were stimulated with the identical peptides at t=0 were pooled and re-split. 100µl of complete aMEM (10% FCS & 0.2µg/ml Brefeldin A) was added 1 hour after peptide restimulation to block secretion of proteins. Following incubation, cells were stained for 15 mins with anti-CD8; in the fridge, fixed and permeabilized with cytoperm/cytofix for 20mins; 4°C, stained with anti-IFN-γ in PBS + 0.01% Saponin and 1% FCS for 30mins, washed (PBS; 1% FBS) and analyzed by flow cytometry. For Bim/Mcl-1 costaining, 0.5µg or 2/100 dilution of Bim and Mcl1 primary antibody was added along with IFN-γ, followed by two washes with PBS/FCS(1%)/Saponin(0.1%). Samples were then stained with the secondary antibody 1/2000 and 1/200 dilution for anti-Bim and anti-Mcl-1 secondaries, for 20 minutes, followed by two washes with PBS/FCS(1%)/Saponin(0.1%) and finally resuspension of the sample in PBS/FCS(1%) for flow cytometry. Antibodies were titrated down to these working dilutions to minimize non-specific cross-reactivity to intracellular antigens or the other antibodies (CD8 and IFN-γ).

2.9 Flow cytometric analysis

Routine flow cytometry was conducted on a FACs calibur. Following staining, cells were fixed with PBS + 1% formaldehyde. Samples were acquired on the same day. A live acquisition gate (R1) was set on viable lymphocytes based on the forward (linear scale) and side scatter (log scale) profile that represents size and granularity respectively. Single stained samples were set-up to compensate for fluorescence variations. A null channel was used to gate out non-specific autofluorescence.

2.12 mRNA extraction

Messenger RNA was extracted with the mRNA Direct Kit (Dynal) according to manufacturers instructions. Briefly, 0.3-0.5 x 10^6 total PBMCs were pelleted at 3,200rpm on a bench-top centrifuge for 6 mins. The pellet was resuspended in 50µl PBS, followed by lysis of cells in 500µl Dynal Lysis buffer. 60µl Dynabeads were reconditioned with lysis buffer and combined with the lysate. Samples were rolled at room temperature for 10 minutes to allow the mRNA to bind to the beads. The beads were then separated magnetically from the lysate, followed by 300µl washes of Wash Buffer A (twice), 300µl Wash Buffer B (twice) and 100µl DEPC water (once). mRNA was eluted twice from the beads into 200µl DEPC water containing 5ng/ml T7-p(T) primer by heating to 95°C for 2 mins followed by magnetic separation, collection of bead-free eluate and reduction of the volume by vacuum centrifugation.

2.13 In vitro amplification

Purified mRNA was amplified twice according to manufacturers instructions (Ampliscribe T7-Flash transcription kit, Epicentre Technologies) and 5µg (quantified with an Agilent Bioanalyser) was Cy5-labelled (Amersham Pharmacia Biotech).

mRNA from 0.3-0.5 x 10^6 PBMCs was reverse transcribed with Superscript II (Gibco) utilizing a Poly T primer containing a RNA polymerase T7 Promoter. Second strand was generated by snap-back priming of the 3' end of the first DNA strand. The double stranded product was purified with phenol:choloroform:isoamyl alcohol (Invitrogen) for protein extraction and Microcon 100 columns (Millipore) for desalting and removal of dNTPs, and reduced to a calculated volume by vacuum centrifugation. This product was linearly amplified with the Ampliscribe T7-Flash transcription kit (Epicentre Technologies, Madison, WI) according to manufacturers instructions, and then purified as described above.

The product of the first amplification (aRNA) was reverse transcribed with random hexamers (Amersham-Pharmacia Biotech., UK), followed by second strand

generation with a T7-p(T) primer. The double stranded product was purified as above, reduced in volume by vacuum centrifugation, linearly amplified, and purified again (see figure 2.1).

2.14 Quantitation of mRNA

Messenger RNA was accurately quantified with a Bioanalyser (Agilent) using NanoChips (Agilent) according to the manufacturers instructions. Briefly, the chip was primed with gel-dye matrix, sample and ladder wells were loaded with marker and finally 1µl of sample was loaded per well (upto 12 samples/chip) along with 1µl (150ng) RNA ladder (Ambion), vortexed and read within 5mins. The ladder was heat-denatured at 95°C and snap chilled on ice prior to loading to remove secondary structures.

2.15 RNA Labelling

5µg of aRNA were labelled with the Cyscribe First Strand cDNA Labelling Kit (Amersham Pharmacia Biotech.) according to manufacturers instructions, with random nonamers and Cy5-dCTP (Amersham) for samples and Cy3-dCTP (Amersham) for reference, followed by purification (Microcon30) and vacuum concentration to the required volume.

2.16 Hybridisation

cDNA arrays (Human cDNA Array Gen2 (Hs_clone_Av2) were provided by the Rosalind Franklin Centre for Genomics Research; further information can be found on : www.rfcgr.mrc.ac.uk/microarray

The Cy5 and Cy3-labelled samples were combined in hybridisation buffer consisting of 12µl x20 SSPE (Sigma-Aldrich), 1.1µl 0.5M EDTA (Sigma-Aldrich), 2µl Poly d(A) (Amersham), 2µl tRNA (Sigma-Aldrich) in a volume of 45µl. 1µl of 10% SDS (Sigma) was then added and the sample was incubated at 98°C for 2 mins, followed by 37°C for 20mins. Finally, 1µl of x100 Denhardt's (Sigma) was added, spun at 13,000rpm for 15mins and dispensed onto an array which was then topped with a coverslip (BDH) and incubated in a humidified chamber (Ambion) at 65°C overnight. Arrays were washed (x2 SSPE at 50°C, x2 SSPE at room temperature, x1 SSPE at

RT, x0.1 SSPE at RT), spun dry (1000rpm; 3mins) and scanned (Axon; Genepix software) at a PMT intensity such that white spot over-saturation was minimized. A lower and higher PMT was also taken in order to be able to evaluate low and highly expressed genes, respectively.

2.17 Primary array analysis

The scanned images were initially gridded with a standard algorithmn and subsequently fine tuned manually with genepix software. Data was then extracted and exported as an excel-compatible file, of which only the following 11 data columns were retained:

Block
Column
Row
Name
ID
F635 Median
B635 Median
F532 Median
B532 Median
Median of ratios (635/532)
Flags

From these, the signal-background for the two fluorescence channels were generated, Log_2 transformed and then the signal to noise ratios (SNR) for Cy3 and Cy5 were calculated. The first row of each of the 48 blocks of spots on each array that contained the control genes were selected and pasted into a new worksheet entitled "Scorecard" and were sorted according to ID. Within these were 15 spots corresponding to negative control genes that included the yeast intergenic region from Chromosome VII, X1, XII and XIII, the arabidopsis thaliana protein G1p, a poly-dA oligonucleotide, spotting buffer and a Bacillius subtilis gene. The mean Cy5 and Cy3

SNR of these 15 negative genes was used as the cut-off to filter out genes with a low SNR thereby only selecting data above the background. Only genes that passed both Cy5 and Cy3 SNRs negative control filtration were selected thereby producing a single column of quantitative numerical data of the relative expression of each gene on the array.

2.18 Cluster/Treeview analysis

Software obtained from : http:/rana.lbl.gov/EisenSoftware.htm.

Manipulation by CLUSTER was performed in 21 stages:

1) The data were compiled into a single excel spread-sheet and negative and positive genes were deleted. The gene expression data (1 to n arrays) was then processed by formatting the excel file as follows:

Gene I.D.	**Name**	**GWEIGHT**	**GORDER**	**DATA SERIES 1**	**DATA SERIES 2**	**DATA SERIES n**
EWEIGHT				1	1	1
EORDER				1	2	n
I.D. of A	A	1	1			
I.D. of B	B	1	1			
I.D. of C	C	1	1			
I.D. of x	x	1	1			

2) save data as a text (delimited) file

3) initiate CLUSTER and load text data file created in step 2

4) adjust data: median center genes and arrays

5) click "APPLY" ten times

6) filter data (%), if required

7) in SELF-ORGANISING MAPS panel, select "organize genes"

8) then "MAKE SOM" (if required)

9) go to excel and open the SOM file created in step 8

10) insert a column after column C

11) enter "=C3*-1" in D3; extend and D3 cell function to all the adjacent cells in the column

12) copy column D

13) paste special into Column C and label it "GORDER"

14) delete column D

15) insert new column titled "GWEIGHT" before GORDER; all cell with a value of 1

16) make all EWEIGHT = 1

17) insert EORDER numbers after EWEIGHT (1,2,3, etc)

18) save as a tab delimited file; include "EDITED" in the name

19) go to CLUSTER

20) load file created in step 18

21) in the hierarchical clustering panel, select average linkage clustering

The final step (22) saves three files in the default folder. The cdt file can be viewed in TREEVIEW; the heat-map, gene dendogram and array dendogram can subsequently be saved as unique image files. The atr and the gtr file contain the array tree and the gene tree respectively and can be viewed in excel.

2.19 Significance analysis of microarrays (SAM)

SAM was obtained from: http://www-stat.stanford.edu/~tibs/SAM/index.html
Gene expression data, normalized in CLUSTER (median centring), was formatted to SAM requirements as indicated in the manual that was downloaded from the web link above.
The basic format was as follows:

		1	1	1	1	1	etc	2	2	2	2	2	etc
Name of gene 1	I.D. of gene 1												
Name of gene 2	I.D. of gene 2												
Name of gene 3	I.D. of gene 3												
Name of gene 4	I.D. of gene 4												
etc	etc												

Array data from resolved samples were pasted in serial columns headed with the response variable "1", followed by array data from chronic samples that was headed "2". Empty data cells were replaced with "NA". All the data cells were then highlighted and the SAM excel add-in was executed. The false discovery rate was set as required when prompted by SAM.

2.20 Real time Quantitative PCR

Approximately 5μg of twice-amplified aRNA from resolved or chronic patient samples was reverse transcribed with superscript II reverse transcriptase and random nonamers in a 25μl reaction volume, according to manufactures instructions. 10% of the reaction was added to an appropriate quantity of Taqman universal PCR master mix and the commercially designed gene-specific primer set that included an internal fluorescent (FAM) probe in a final volume of 25μl according to the manufacturers instructions and run on an ABI prisim 7000 (Applied Biosystems). For normalization purposes, each sample was set up in duplicate; one contained the Bim primer/probe set and the second contained the internal housekeeping gene (HuPo); the latter was kindly provided by J.Huggett (U.K.); both reactions had an equal amount of cDNA template. The relative quantification value was calculated as recommended by Applied Biosystems, as follows: ΔCT (Bim) - ΔCt (HuPo) = ΔΔCT, and 2 - ΔΔCt = relative quantification value.

2.21 Reagents

Agilent Biochip biochips/reagent	Agilent	50654476
aMEM	Gibco	22571020
Bax inhibitor (VPMLK)	Proimmune	POG
Bim Assay on Demand	Applied Biosystems	Hs00197982
b-Mercaptoethanol	Gibco	31350010
Brefeldin A	Sigma	B7651
CMV Peptide	Proimmune	POBG008
Core 18-27 pentamer	Proimmune	FOAG023

Core 18-27 Peptide	Proimmune	POBG 023
Cot1-DNA	Amersham	15279-011
Cy3- dUTP	Amersham Pharmacia	PA53021
Cy5- dUTP	Amersham Pharmacia	PA55021
Cyscribe labelling kit	Amersham Pharmacia	RPN-6200
Cytoperm/Cytofix	Becton Dickinson	554722
Denhardts (x100)	Sigma	D9905
DEPC-treated water	Invitrogen	10977.015
DMSO	Sigma	D4540
DNA polymerase I	Gibco	18010025
dNTP	Amersham	27203501
EDTA	Sigma	E7889
Envelope 183-191 pentamer	Proimmune	FOAG027
Envelope 183-191 Peptide	Proimmune	POBG 027
Essential amino acids	Gibco	11130.036
Fetal bovine serum	Invitrogen	10108165
Ficoll	Sigma	H8889
Formaldehyde	Sigma	F8775
Hepes	Gibco	15630056
Human cDNA Microarrays	Rosalind Franklin Centre for Genomic Research	
HuPo Assay on Demand	Applied Biosystems	
IFN-γ secretion assay	Miltenyi Biotech	130-054-202
IL-2	Roche	1011456
Influenza peptide	Proimmune	P007.0A
Ionomycin	Sigma	I0634
Microcon 100	Millipore	42413
Microcon 30	Millipore	42410
mRNA Direct Kit	Dynal	61021
Nano RNA600 chips	Agilent	50654476
Nonessential amino acids	Gibco	11140.035
Penicillin/streptomycin	Invitrogen	15070.063
Phenol/Chloroform/Isoamyl alcohol	Invitrogen	10977015

Phorbol 12-Myristate 13-Acetate	Sigma	P8139
Poly A(40-60)	Amersham	27.7988.01
QPCR Master mix	Applied Biosystems	4304437
Random nonamers	Sigma-Aldrich	R7647
RNA Ladder	Ambion	7152
RNase Zap	Ambion	9780
RNasin	Promega	N2111
RPMI	Autogen Bioclear	NG52052
Second strand buffer	Gibco	10812014
Sodium pyruvate	Gibco	11360.039
SSPE (x20)	Sigma	S2015
Superscript II	Invitrogen	18064.014
T4 DNA polymerase	Gibco	18005025
T7 transcription kit	Epicentre	ASF3257
Taqman Universal mastermix	Applied Biosystems	4304437
Universal reference RNA	Stratagene	740000
Yeast tRNA	Sigma	R8759
zVAD-fmk	BD	550337

2.22 Antibodies

Anti-PE microbeads	Miltenyi Biotech	130-048-801
BimEL/L/S (rat anti human)	Axxora	ALX.804.527
CD127-PE	BD	557938
CD3 PerCpCy5.5	BD	332771
CD8+ PE-Cy5	BD	555368
CD8+ APC	BD	555369
Goat anti rabbit FITC	Insight Biotech	SC-2012
Goat anti Rat FITC	Axxora	A110-109F
HLA-A2 FITC	serotec	MCA2090F
IFN-γ PE	R&D	IC285P
Mcl-1 (Rabbit anti Human)	Insight Biotech	1239-1

3 CHAPTER 3

An analysis of the breadth of the HBV-specific CD8+ T cell response in individuals with resolved and chronic HBV infection.

3.1 Background

Virally infected cells within a host need to be rapidly identified and the pathogen eradicated. MHCI complexes display fragments of viral antigens for recognition by CD8+ T cells that bear complementary receptors (TCRs) (Yewdell and Bennink, 1992). Hundreds to thousands of antigenic peptide fragments can potentially be generated from viral proteins (Yewdell, 2006) but the CD8+ T cell response that is evoked is thought to be focused on a limited number of viral determinants. However, data generated using more comprehensive up-to-date analytical techniques suggests that the antiviral hierarchy may actually be larger than previously thought (Posnett et al., 2005; Sacre et al., 2005). Nonetheless, these responses can be very conserved across individuals with a similar MHC background (Chen et al., 2000; Fuller et al., 2004).

In a primary infection, several factors govern the development of this hierarchy. These include the efficiency of antigen presentation, the stability of the viral peptide-MHCI complex on the surface of professional antigen presenting cells, the presence of a population of naïve CD8+ T precursors with the ability to recognize the specific peptide, and a phenomenon termed immunodomination where certain specificities influence the expansion of others (Kedl et al., 2003; Newberg et al., 2006; Roy-Proulx et al., 2001). Additionally, heterologous immunity could influence a primary response because cross-reactive memory CD8+ T cell populations of unrelated specificities could expand faster than naïve CD8+ T cells; these would therefore dominate over simultaneously primed but slower-to-expand naïve cells (Brehm et al., 2002; Chen et al., 2001; Selin and Welsh, 2004). This latter factor contributes to the individual's private immune hierarchy and depends on each individual's history of exposure to pathogens (Welsh and Selin, 2002).

The focus of this work relates to several studies that have shown that persistent viral infection is associated with an immunodominance hierarchy that is different to that established during acute infection (Bergmann et al., 1999; Goulder et al., 2001; Hislop et al., 2002; Tewari et al., 2004; Wherry et al., 2003). This suggests that perturbations of the response could be responsible for immune failure. Multispecificity is important to effective viral control for several reasons. Targeting multiple viral epitopes exerts greater (simultaneous) pressure against the virus. This is particularly critical to pathogens that attempt to escape through mutation. Certain specificities may be better (faster) than others at expanding; this could be due to the inherent capacities of the cells involved (Hill, 1999). Assessing the differences between virus-specific CD8+ T cell hierarchies associated with good and poor outcomes could help us better understand the manner by which the pathogen establishes chronic infection. Although several groups have made valuable contributions, the majority of the developments on this theme have derived from murine experiments.

Hepatitis B virus infection presented an ideal opportunity to study virus-specific CD8+ T cell hierarchies in humans because of the natural dichotomous outcome where some patients successfully spontaneously resolve infection but others remain persistently infected. Several years ago, progress in the study of the HBV-specific CD8+ T responses was hampered by the inability to propagate the virus *in vitro* but investigations with primary HBV infected tissue confirmed an active participation of these immune cells. The subsequent characterization of HLA-A2 binding synthetic peptides (Sette et al., 1994) facilitated the identification, isolation and analysis of multiple HBV-specific CD8+ T populations. Further studies demonstrated that effective viral control was associated with robust HBV-specific CD8+ T responses (Bertoni et al., 1997; Nayersina et al., 1993; Rehermann et al., 1996a; Rehermann et al., 1995a). A direct causative effect was then demonstrated by selective depletion of CD8+ T cells in HBV infected chimpanzees confirming that this compartment contained the critical effector cells that enabled containment of the virus (Thimme et al., 2003).

In humans, these HBV-specific populations were found to persist for decades following resolution of the infection (Penna et al., 1996; Rehermann et al., 1996a) and immunosuppression has been associated with the resurgence of HBV replication suggesting that inactive reserves of virus remain and memory sentinel populations, that maintain the capacity to exert effective functional activity, keep the pathogen contained.

In striking contrast to individuals that successfully limit infection, those that are unable to exert effective control but instead maintain varying degrees of viral replication are typically associated with relatively weak or non-detectable CD8+ T responses (Rehermann et al., 1995b), but resolution following chronic infection has been associated with an increase in the virus-specific populations (Lohr et al., 1995; Marinos et al., 1995; Rehermann et al., 1996b), further supporting the evidence for their involvement to be a prerequisite to successful viral control.

Structural and non-structural HBV proteins are both highly immunogenic (Bertoletti et al., 1991; Nayersina et al., 1993; Rehermann et al., 1995a) but relatively little is known about the immunogenic or tolerogenic hierarchies of these individual responses in humans. Although animal studies have generated important data, the main weakness of the use of a transgenic mouse model of persistent HBV infection (Kakimi et al., 2002) is that it is a biological system that is very different to that of humans. The difference of MHC composition between mice and men is of particular importance; antigen processing, CD8+ T cell priming and precursor frequencies are key factors that are not comparable and significantly impact the immune hierarchies formed.

The existing human investigations on this subject indicate that the acute phase CD8+ T response to HBV in resolvers is multi-specific whereas it is oligoclonal in chronic infection (Nayersina et al., 1993; Rehermann et al., 1995a), but the critical weakness of these studies is that they were conducted with limiting dilution/chromium release assays. Recent developments in techniques for detecting virus-specific CD8+ T cells

have demonstrated that older methods have greatly under-estimated the true *in vivo* frequencies of the antigen-specific populations (Butz and Bevan, 1998; Maini et al., 1999; Murali-Krishna et al., 1998). This was partially due to the inherent technical limitations that only allowed the detection of cells that maintained *in vitro* proliferative and functional (lytic) capacity. It is now clear that lengthy *in vitro* culture with multiple rounds of peptide stimulation would have rendered the majority of the antigen-responsive CD8+ T cells highly susceptible to activation-induced cell death (Lenardo et al., 1999).

Novel technologies have now largely replaced older assays, a major advance being the development of fluorescently labelled peptide-loaded HLA multimers such as tetramers, pentamers, and nonamers. When applied to flow cytometry, these reagents allow a highly accurate quantitation and analysis of the antigen-specific CD8+ T cell populations.

Our group has successfully applied these new techniques for direct *ex vivo* analyses of HBV polymerase, core, and envelope-specific CD8+ T in a group of 23 acute resolvers (Maini et al., 1999) and we have also been able to examine the TCR usage of core-specific CD8+ T cells in resolved and persistently infected individuals (Maini et al., 2000b). However, only a limited panel of viral determinants were assessed and a more comprehensive analysis to better understand immune hierarchies associated with resolved and chronic HBV infection was required.

At the time, we also demonstrated that certain individuals with chronic high viral replication maintained a peculiar population of HBV envelope-specific CD8+ T cells that were unable to bind HLA-matched tetramers (Reignat et al., 2002); this phenomenon has been described previously in association with T cell dysfunction (Blohm et al., 2002; Demotte et al., 2002; Rubio-Godoy et al., 2001; Spencer and Braciale, 2000). The discovery of this phenotypic defect invalidated the use of tetramers for a more comprehensive study of the breadth of the antiviral CD8+ T cell response in individuals with chronic infection, particularly as it was not clear whether other specificities were also prone to this defect. However, as tetramer-negative

CD8+ T cells maintained the ability to produce IFN-γ following *in vitro* peptide-specific stimulation, we were able to apply the technique of intracellular cytokine staining (ICS) to identify these specificities (Jung et al., 1993). Although the functional capacity of effector CD8+ T cells can be compromised in chronic viral infection, this occurs sequentially. It begins with the loss of IL-2 production, followed by the inability to lyse target cells that then leads to the loss of TNF-α production. The inability to produce IFN-γ is the most resistant and this defect occurs last (Wherry et al., 2003).

Nonetheless, it is still extremely difficult to detect HBV-specific CD8+ T cells in peripheral blood by ICS for IFN-γ directly *ex vivo*, but the frequencies of these scarce populations can be significantly enhanced by short-term *in vitro* enrichment. Although these methods cannot be used to determine the true *in vivo* frequencies, the trends observed after enrichment are representative of the patterns observed directly *ex vivo* (Webster et al., 2004).

Thus, in order to avoid overlooking low-frequency specificities due to the limitations of the direct *ex vivo* assay, we chose to maximise our capacity to detect different specificities by applying short-term *in vitro* enrichment. By forfeiting the acquisition of data on *in vivo* frequencies within the range of detection we aimed to obtain a more representative profile of the overall specificity of the response that was present in each individual studied.

Persistent HBV infection of humans manifests as a highly heterogenous disease (Fattovich, 2003); however, individuals can be broadly divided into two groups. There are those that have low levels of viral replication (as determined by the quantity of infectious viral particles in the serum) that is indicative of partial but inefficient viral control. Alternatively, there are individuals with high levels of viral replication suggesting a totally compromised immune response. HBeAg antigen, the secreted form of the N-terminal elongated core protein, was commonly used as a serological indicator of this latter phase of infection but an increase in the incidence of a strain containing a precore mutation resulting in the loss of e antigen secretion

has now invalidated this approach. Currently, viral load is the most representative indicator of the status of viral replication.

As differences in the quantity and quality of the CD8+ T cell compartment of individuals with low or high levels of chronic infection could influence the overall antiviral efficacy it was important to stratify these individuals into two groups. Thus, in addition to examining the hierarchy in the resolved patients (HBV DNA negative & anti surface antigen antibody positive), we studied persistently infected individuals following their segregation into one of two groups: low and high level of viral replication divided according to a serum viral load of $<10^7$ and $>10^7$ copies/ml, as has been previously reported (Webster et al., 2004).

Thus, the principal aim of this study was to assess the quality of the antiviral HBV-specific CD8+ T cell response by determining the breadth of the antigen specific response in individuals with good, intermediate and poor outcomes. Our data have confirmed and extended previous knowledge on the association of HBV-specific antiviral specificities to different virological outcomes.

3.2 Results

3.2.1 Detection of hepatitis B virus core-specific CD8+ T cells

HBV-specific CD8+ T cells are difficult to identify in the peripheral blood of resolved individuals because the frequency of circulating cells are very low; these are even more scarce in chronic infection. A short-term enrichment strategy allowed us to overcome this problem. Figure 3.1 is a representative example of the staining typically observed with cells taken from an individual that resolved HBV infection. The cells were enriched over 10 days for core 18-27-specific CD8+ T cells. In this example, the direct *ex vivo* virus-specific CD8+ T cell frequency was found to be 0.5% of total CD8+ T cells (figure 3.1a). Enrichment by short-term culture increased the in vivo peripheral frequency to 20% of total CD8+ T cells (figure 3.1b), corresponding to an increase in the antiviral population from 1 in 1000 (*ex vivo*) to 40 in 1000 total PBMCs (given that the CD8+ T cell compartment amounted to 20% of the gated cells). Unstimulated cells were also stained simultaneously and used to evaluate the non-specific background; PMA/ionomycin treated cells allowed us to evaluate the functional capacity of the sample following 10 days of culture (figure 3.2). This latter condition also allowed us to determine the fidelity of the overall staining procedure.

Importantly, one of the original aims of this work was to attempt to apply Genechip technology to study the transcriptional profiles of HBV-specific CD8+ T cell populations. *In vitro* enrichment enabled us to generate the numbers of cells required for these assays; enriched samples were more likely to exhibit transcriptional profiles reflective of the virus-specific effector population (when activated) rather than material sampled directly *ex vivo* where these populations would only be a very small fraction of the total.

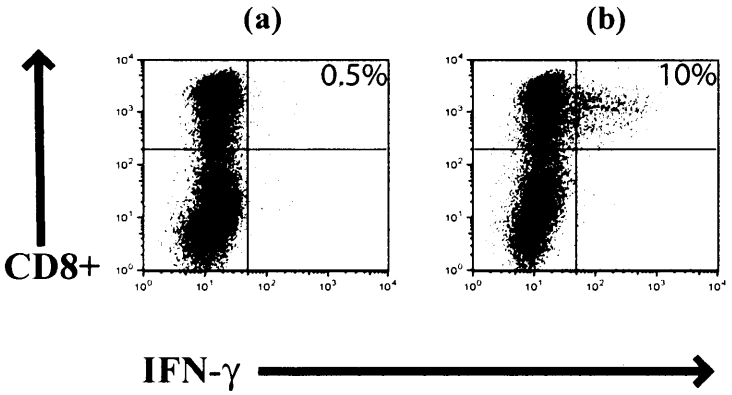

Figure 3.1
Detection of HBV core-18-27-specific CD8+ T cells directly *ex vivo* and following short-term enrichment
Total PBMCs from a representative resolved individual were stimulated with peptide for 6 hours directly *ex vivo* (a) or following 10 day expansion (b) in the presence of Brefeldin A and stained for surface CD8 and intracellular IFN-γ.

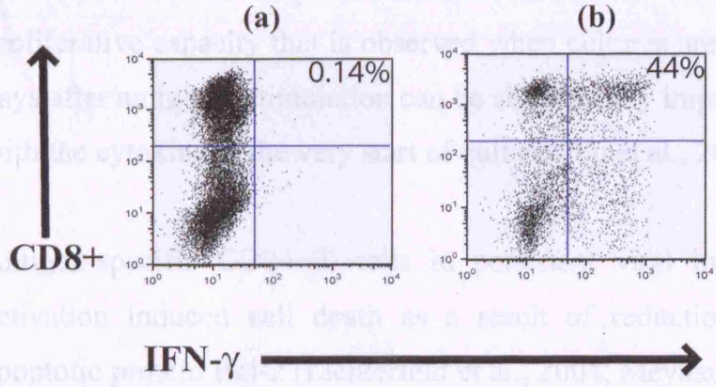

Figure 3.2
Negative and positive controls
Unstimulated cells (a) and TCR-independent stimulation with PMA/Ionomycin (b) was used to evaluate the non-specific background and the general functional capacity of the short-term lines.

3.2.2 Optimisation of virus-specific CD8+ T cell enrichment

The standard protocol for *in vitro* enrichment of HBV-specific CD8+ T cells involved supplementation of the growth medium with recombinant IL-2 on the fourth day of culture. Earlier methods included recombinant HBV core protein in the primary stimulation in order to harness endogenous CD4 T cell help to maximise the efficiency of proliferation (Missale et al., 1993). Cross presentation could have driven the expansion of undesired specificities by providing alternative core-derived peptides, and thus core antigen was not used in our studies.

Recent work on HIV-specific CD8+ T cells have demonstrated that a sub-optimal proliferative capacity that is observed when cultures are supplemented with IL-2 two days after antigenic stimulation can be significantly improved if the cells were treated with the cytokine at the very start of culture (Yu et al., 2006).

Antigen-specific CD8+ T cells in persistent viral infection are highly prone to activation induced cell death as a result of reductions of the intracellular anti-apoptotic protein Bcl-2 (Lichterfeld et al., 2004; Meyaard et al., 1992; Petrovas et al., 2004). However, other work has shown that IL-2 receptor signalling can initiate Bcl-2 expression through Stat5 signalling (Lord et al., 2000). It can also encourage cellular proliferation by affecting cyclin D2 (Martino et al., 2001) and the proto-oncogenes c-myc or lck (Miyazaki et al., 1995). These data indicated that by treating cells with IL-2 at the time of stimulation, it could be possible to antagonize intrinsic mechanisms that normally contribute to the death of the activated cells and well as positively influence their proliferation.

We therefore sought to further optimise the virus-specific enrichment by modifying the IL-2 treatment regime. In order to conserve our limited patient samples, we conducted these studies on human cytomegalovirus (HCMV)-specific CD8+ T cells. HCMV belongs to the herpes viruses family and typically establishes a low-load persistent lifelong infection (Klenerman and Hill, 2005). Up to 70% of the population

in economically advanced countries have been exposed to this virus and mount a successful immune response to it (Harari et al., 2004; Maecker and Maino, 2004). An immunodominant CD8+ T cell determinant of the CMV protein pp65 had been described (Wills et al., 1996) and allowed us to rapidly identify and study this response in peripheral venous blood taken from healthy laboratory volunteers.

PBMCs from a healthy HLA-A2 positive individual (previously determined to possess CMV-specific CD8+ T cells) were stimulated with an HLA-A2 restricted CMV peptide (NLVPMVATV) on day 0. IL-2 was added to the growth medium in three variations: at the start of culture only, at day 4 only or at both day 0 and day 4. The success of the expansion under these different conditions was determined by re-stimulating the cultures on day 10 with the specific CMV-peptide followed by staining for intracellular IFN-γ. Unstimulated cells that were treated with IL-2 on day 0 provided a negative control.

Following the standard protocol, we were able to detect CMV-specific CD8+ T cells at a frequency of 0.84% (of total CD8+ T cells) in stimulated PBMCs exposed to IL-2 on day 4 only. However, those that received IL-2 at the start of culture had a much larger population of 2.05% (of total CD8+ T cells). Furthermore, an even greater frequency of 2.47% (corresponding to an overall increase of 294%) was observed by the addition of IL-2 both at the onset of stimulation as well as on day 4 (figure 3.3). Similar influences following temporal variations of IL-2 treatment were obtained for HBV-specific CD8+ T cell expansions (personal communication with A.Bertoletti, Singapore) and therefore this modified protocol was incorporated into the study.

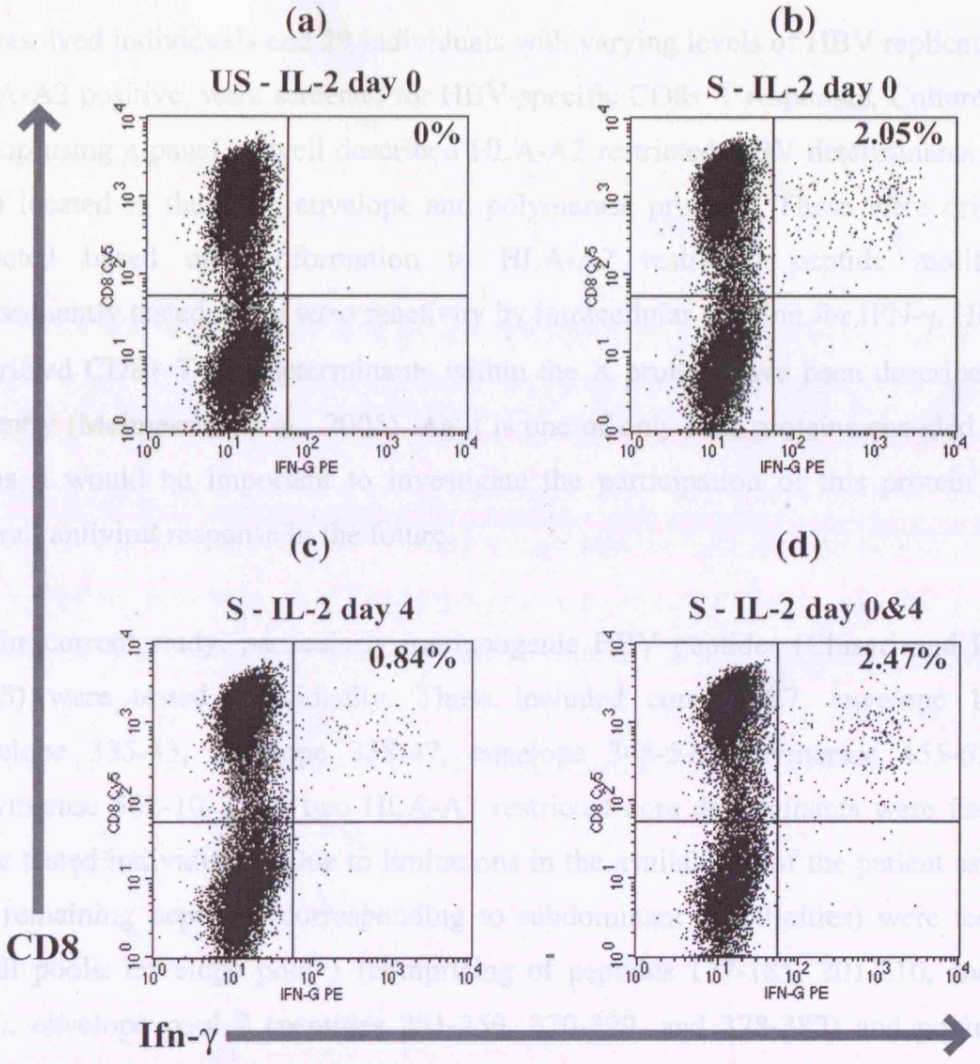

Figure 3.3
Influence of the time of IL-2 supplementation on the expansion of virus-specific CD8+ T cells
Total PBMCs from an HLA-A2 positive healthy donor were cultured with an HLA-A2 restricted CMV peptide for ten days. IL-2 was supplemented into the growth medium at the onset of culture (b), on day four (c) or at both the onset and on day four (d). Unstimulated cells treated with IL-2 on day zero served as a negative control (a). Samples b, c and d were restimulated with CMV peptide on day 10 followed by staining of all four samples, with specific monoclonal antibodies, for surface CD8+ and intracellular IFN-γ. Frequencies of virus-specific cells (as a percent of total CD8+) are indicated in the top left quadrant.

3.2.3 CD8+ T cell responses to HBV core, envelope and polymerase

10 resolved individuals and 29 individuals with varying levels of HBV replication, all HLA-A2 positive, were screened for HBV-specific CD8+ T responses. Cultures were set up using a panel of well-described HLA-A2 restricted HBV determinants (Table 3.1) located in the core, envelope and polymerase proteins. These were originally selected based on conformation to HLA-A2 restricted peptide motifs and subsequently tested for *in vitro* reactivity by intracellular staining for IFN-γ. HLA-A2 restricted CD8+ T cell determinants within the X protein have been described only recently (Malmassari et al., 2005). As it is one of only four proteins encoded by the virus it would be important to investigate the participation of this protein in the overall antiviral response in the future.

In the current study, particularly immunogenic HBV peptides (Chisari and Ferrari, 1995) were tested individually. These included core 18-27, envelope 183-91, envelope 335-43, envelope 338-47, envelope 348-57, polymerase 455-63, and polymerase 502-10. Only two HLA-A2 restricted core determinants were used and were tested individually. Due to limitations in the availability of the patient samples, the remaining peptides (corresponding to subdominant specificities) were tested in small pools: envelope pool 1 (comprising of peptides 177-185, 201-210, and 204-212), envelope pool 2 (peptides 251-259, 370-379, and 378-387) and polymerase pool 1 (peptides 575-583, 655-663, and 816-824).

As stated previously, chronic HBV infection is a very heterogenous disease and therefore this group of individuals were segregated into those that exhibit partial viral control (low viral load) and others that are unable to contain the virus (extremely high viral load). Therefore, patients in the overall study sample fell into one of three groups: group one (resolved) consisted of individuals that had undetectable virus in the serum and were HBsAg negative and anti-HBsAg antibody positive; classical indicators of effective viral control. The individuals with chronic infection were

assigned to either group two (chronic low; with HBV DNA $<10^7$ copies/ml) or group three (chronic high; HBV DNA $>10^7$ copies/ml).

Figure 3.4 is a representative example of the HBV-specific CD8+ T cell staining that was typically observed in group one, two, and three (column 1, 2, and 3 respectively) in response to stimulation with an immunogenic determinant from each of the three major HBV proteins: core, envelope, and polymerase (rows b, c, and d respectively).

We found that the relative size of the responses to all three HBV proteins were highest in the resolved individuals but it appeared that these waned as the viral load increased; this was particularly obvious for the core response, followed by the polymerase response. Core and polymerase-specific CD8+ T cels were not detectable in the patients with viral loads $>10^7$ copies/ml (with the exception of only one patient that exhibited reactivity to a single polymerase peptide). Robust envelope-specific CD8+ T cells however, could not only be detected in the majority of the resolvers but populations of significant magnitude were also found in chronic infection and even in several individuals in the third group that had extremely high viral loads.

Table 3.1: Synthetic peptides used.

Virus	Peptide code	Protein	Location	Sequence
HBV	c18	Core	18-27	FLPSDFFPSV
HBV	c107	Core	107-115	CLTFGRETV
HBV	p455	Polymerase	455-463	GLSRYVARL
HBV	p502	Polymerase	502-510	KLHLYSHPI
HBV	p575	Polymerase	575-583	FLLSLGIHL
HBV	p655	Polymerase	655-663	ALMPLYACI
HBV	p816	Polymerase	816-824	SLYADSPSV
HBV	e177	Envelope	177-185	VLQAGFFLL
HBV	e183	Envelope	183-191	FLLTRILTI
HBV	e201	Envelope	201-210	SLNFLGGTTV
HBV	e204	Envelope	204-212	FLGGTPVCL
HBV	e251	Envelope	251-259	LLCLIFLLV
HBV	e260	Envelope	260-269	LLDYQGMLPV
HBV	e335	Envelope	335-343	WLSLLVPFV
HBV	e338	Envelope	338-347	LLVPFVQWFV
HBV	e348	Envelope	348-357	GLSPTVWLSV
HBV	e370	Envelope	370-379	SIVSPFIPLL
HBV	e378	Envelope	378-387	LLPIFFCLWV
CMV	CMV	pp65	N/A	NLVPMVATV

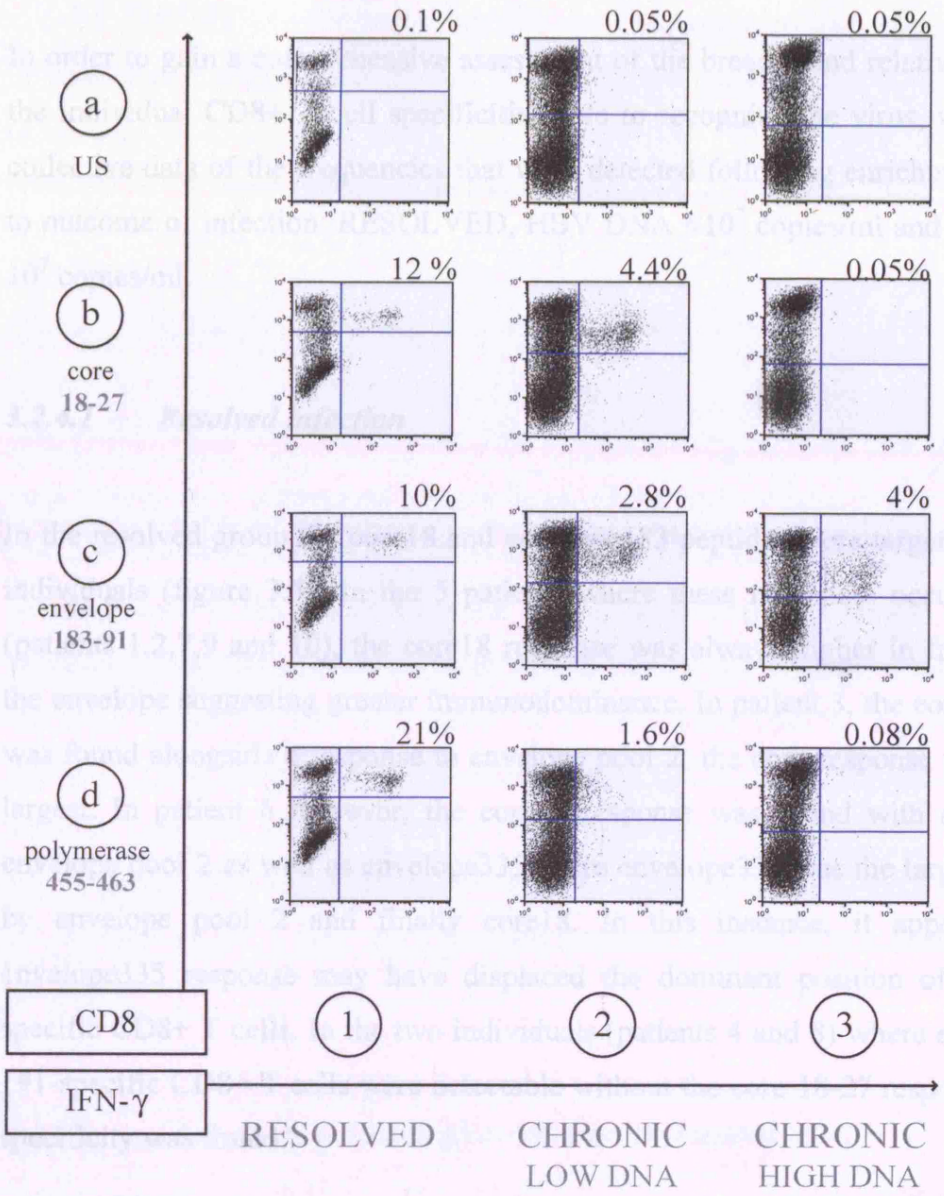

Figure 3.4

Flow cytometric detection of virus-specific CD8+ T cell responses to HBV core, envelope and polymerase proteins.

Total PBMCs from resolved and chronically infected individuals were stimulated with HBV core (18-27), envelope (183-91) or polymerase (502-510) peptides (row b, c and d respectively). IL-2 was supplemented into the medium at the onset of culture and on day four. Cells were restimulated (except negative controls - row a) with relevant peptide on day 10 and then stained for surface CD8 and intracellular IFN-γ. Patients were clustered into three groups [resolved, chronic low DNA (HBV <10 million copies/ml) or chronic high DNA (HBV > 10 million copies/ml)]; column one, two and three respectively. Frequencies of virus-specific cells (as a percent of total CD8+ T cells) in three representative patients are indicated in the upper right quadrant.

3.2.4 Specificity of antiviral CD8+ T cells

In order to gain a comprehensive assessment of the breadth and relative strengths of the individual CD8+ T cell specificities able to recognize the virus, we plotted the collective data of the frequencies that were detected following enrichment according to outcome of infection: RESOLVED, HBV DNA $<10^7$ copies/ml and HBV DNA $>$ 10^7 copies/ml.

3.2.4.1 Resolved infection

In the resolved group the core18 and envelope183 peptides were targeted by 7 of 10 individuals (figure 3.5). In the 5 patients where these responses occurred together (patients 1,2,7,9 and 10), the core18 response was always higher in frequency than the envelope suggesting greater immunodominance. In patient 3, the core18 response was found alongside a response to envelope pool 2; the core response was again the largest. In patient 6 however, the core18 response was found with a response to envelope pool 2 as well as envelope335. Here envelope335 was the largest, followed by envelope pool 2 and finally core18. In this instance, it appears that the envelope335 response may have displaced the dominant position of core 18-27-specific CD8+ T cells. In the two individuals (patients 4 and 8) where envelope 183-191-specific CD8+ T cells were detectable without the core 18-27 response, no other specificity was found.

The most significant polymerase response from the panel that we tested was directed against polymerase455, however, this occurred in only two individuals (patients 2 and 9) but were of significant magnitude. In patient 2 it was smaller than core18 but larger than envelope183; responses to envelope335, 338 and 348 were also found in this person, typifying the multi-specificity of the antiviral response in this patient group. In patient 9, the polymerase 455-463 response dominated over core 18-27 and envelope 183-191; no other specificities were detected. Responses to the polymerase

pool were also detectable in patients 5 and 7; they were independent of other specificities in patient 5 but were the smallest of several others in patient 7. Thus, it appears that the polymerase response can on occasion rise above the general immunodominace of the core 18-27 response that is normally considered to be the dominant HLA-A2 restricted HBV determinant, emphasizing the complexity involved in the establishment of immunodominance hierarchies.

3.2.4.2 Chronic infection (low level viral replication)

Within the group with HBV DNA $<10^7$ copies/ml (figure 3.6), core18 was still the most frequently recognised determinant (7/18 individuals; 39%) followed by envelope183 (4/18 individuals; 22%) and envelope338 (3/18 individuals; 17%). Core 18-27-specific CD8+ T cell were the only type detectable in patient 8, 12 and 17. When this response was detected alongside others, the position within the overall hierarchy varied; it was the highest in 2 individuals (patient 3 and 10), second smallest in one (patient 13) and smallest in one (patient 15). The envelope 183-191 response appeared alongside a core 18-27 response in only one individual (patient 10), and was lower in frequency. In the three other individuals where envelope 183-191-specific CD8+ T cells were detected (patients 2, 7 and 14), it was the dominant specificity.

3.2.4.3 Chronic infection (high level viral replication)

In the group with HBV DNA$>10^7$ copies/ml (figure 3.7), 4/11 individuals demonstrated responses to envelope183; no other accompanying specificity was observed. One patient mounted a relatively small response to envelope pool 1 (e177-185, e201-210, and e204-212). Polymerase502 was recognised by 3% of total CD8+ T cells but in only one individual. None of the 11 patients studied mounted any response to core.

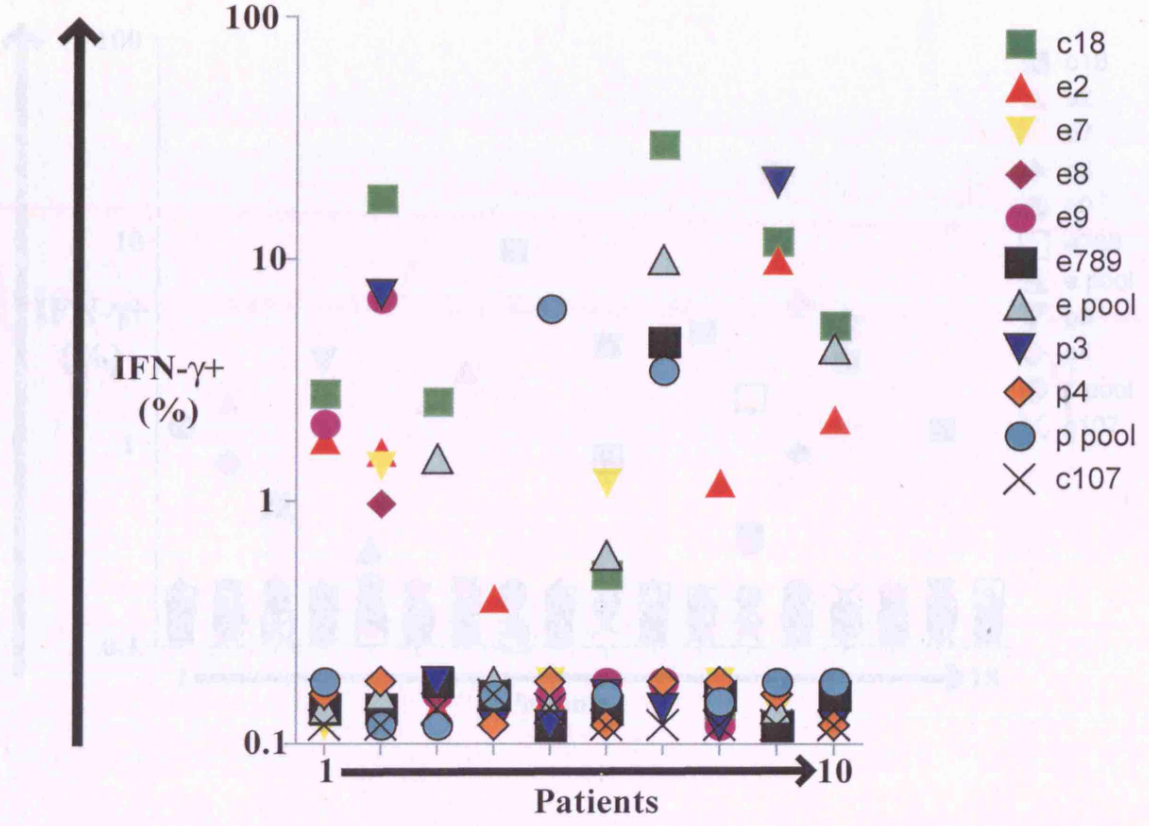

Figure 3.5
Frequency of HBV-specific CD8+ T cell responses in resolved individuals
Short-term lines from resolved individuals restimulated with appropriate peptides and stained for CD8 and IFN-γ. Frequencies correspond to IFN-γ+ of the total CD8+ T cell population.

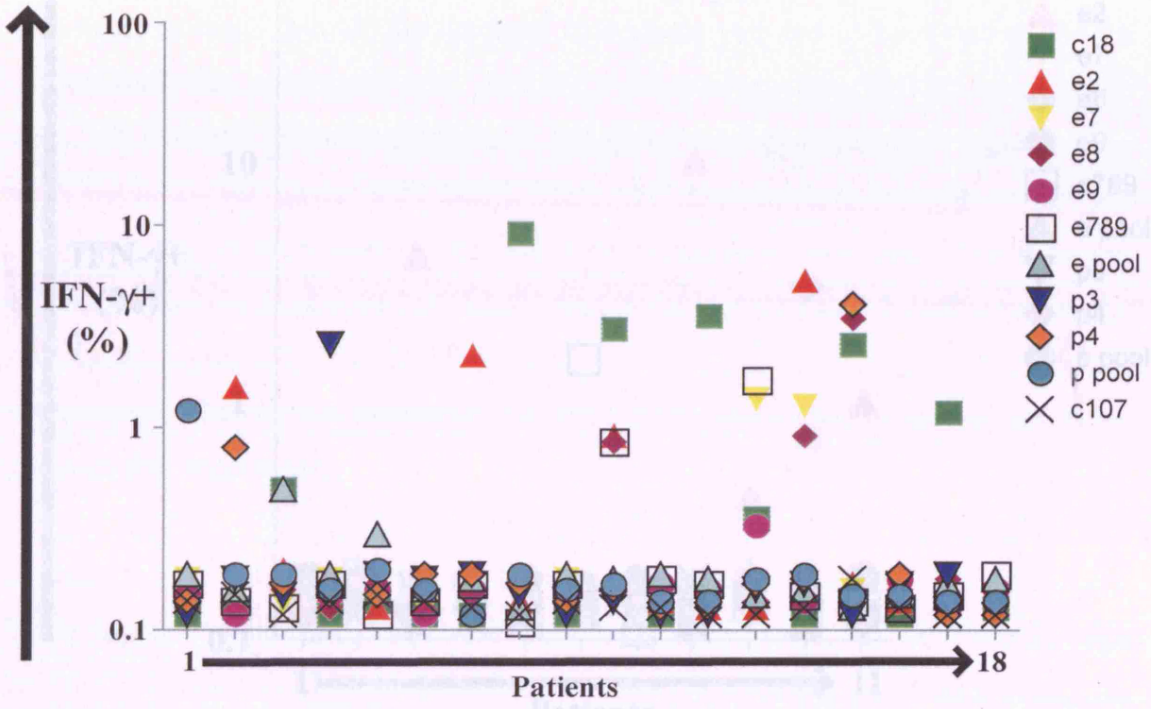

Figure 3.6
Frequency of HBV-specific CD8+ T cell responses in chronic HBV infection (<10mil.copies/ml)
Short-term lines from persistently infected individuals restimulated with appropriate peptides and stained for CD8 and IFN-γ. Frequencies correspond to IFN-γ+ of the total CD8+ population.

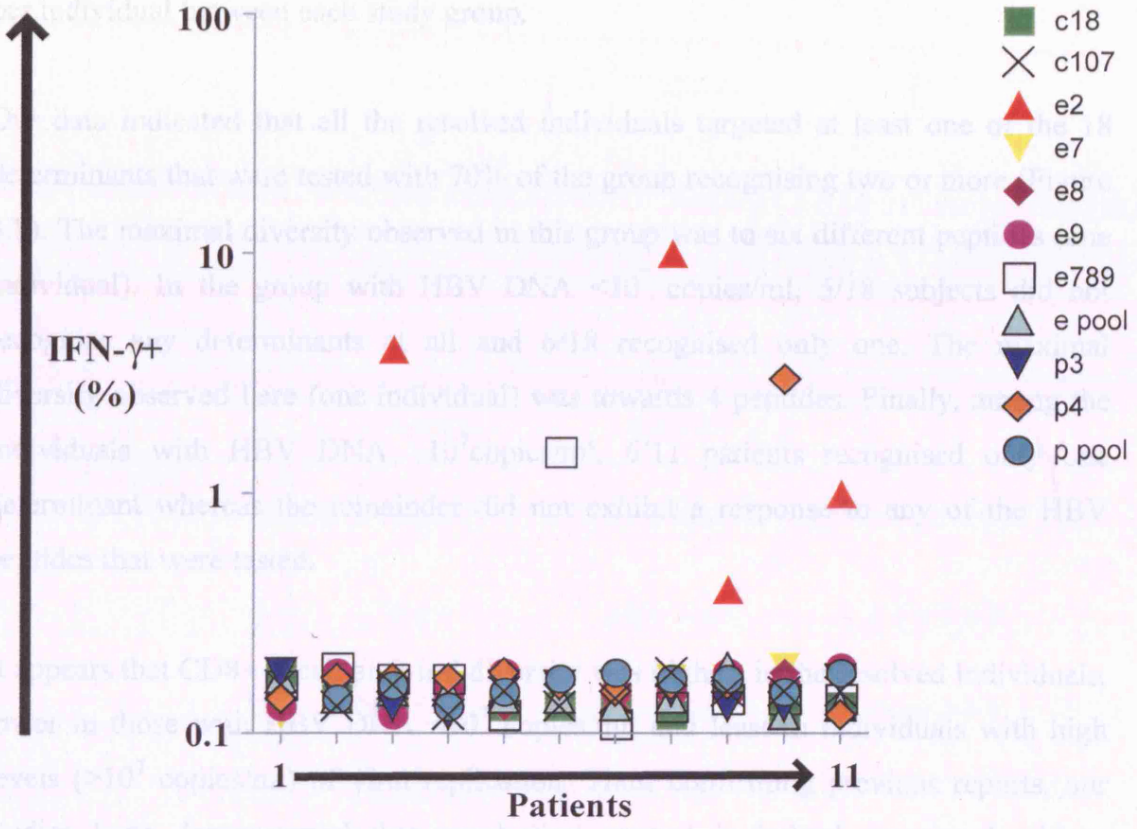

Figure 3.7

Frequency of HBV-specific CD8+ T cell responses in chronic HBV infection (>10mil.copies/ml)

Short-term lines from persistently infected individuals restimulated with appropriate peptides and stained for CD8 and IFN-γ. Frequencies correspond to IFN-γ+ of the total CD8 T cell population.

3.2.5 Diversity of viral determinants targeted by CD8+ T cells

Apart from the individual determinants that were targeted within the three groups studied, we also wanted to evaluate the overall diversity of the antiviral specificity per individual between each study group.

Our data indicated that all the resolved individuals targeted at least one of the 18 determinants that were tested with 70% of the group recognising two or more (Figure 3.8). The maximal diversity observed in this group was to six different peptides (one individual). In the group with HBV DNA $<10^7$ copies/ml, 5/18 subjects did not recognise any determinants at all and 6/18 recognised only one. The maximal diversity observed here (one individual) was towards 4 peptides. Finally, among the individuals with HBV DNA $>10^7$copies/ml, 6/11 patients recognised only one determinant whereas the remainder did not exhibit a response to any of the HBV peptides that were tested.

It appears that CD8+ T cell antiviral diversity was highest in the resolved individuals, lower in those with HBV DNA $<10^7$ copies/ml, and least in individuals with high levels ($>10^7$ copies/ml) of viral replication. Thus, confirming previous reports, our studies have demonstrated that good viral control is indeed associated with a multispecific CD8+ T cell response, and the narrowing of this immune compartment is associated with the loss of viral control.

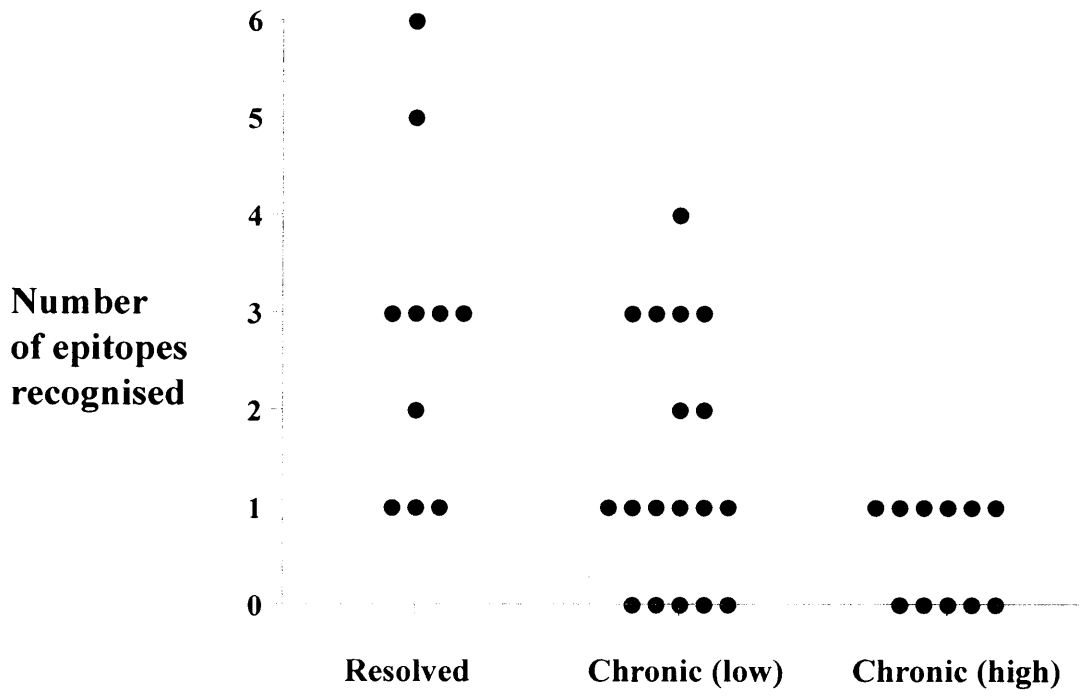

Figure 3.8
Diversity of the CD8+ T cell response to HBV
The number of different HBV-specific epitopes targeted by CD8+ T cells after short term enrichment with relevant HBV peptide is demonstrated in patients segregated according to viral load: resolved (undetectable DNA), chronic low DNA (<10 million copies/ml) and chronic high DNA (> 10 million copies/ml).

3.2.6 HBV-proteins targeted by the antiviral CD8+ T response

We next sought to investigate whether there could be an association between virological status and the particular HBV protein that was targeted. To do this, we calculated the cumulative response for each protein (i.e. the sum of the antiviral frequencies that were observed against the individual determinants within the core, envelope and polymerase proteins) and segregated these data according to virological outcome as described previously (figure 3.9).

We observed that 9/10, 7/10, and 4/10 of the individuals in the resolved group mounted significant responses to envelope, core, and polymerase respectively. The high incidence of envelope (90%) may be a reflection of the relatively large number of determinants of this protein that were tested in this study (11 in total), however, the core protein was represented by only two determinants yet it was targeted in more individuals than was polymerase which was represented by five. Thus, apart from the differing ratio of the individual determinants selected, the relative quantity of each protein could be dictating their relative immunogenicity – polymerase, although required for the earliest steps in replication, is produced in trace quantities compared to the structural proteins and this would bias the T cell priming towards envelope and core-specificities. Nonetheless, as anti-polymerase CD8+ T cells can still be observed, it indicates that factors other than the relative gross quantity influences the immune hierarchy.

In the intermediate group (HBV DNA $<10^7$ copies/ml) 7/18 individuals (39%) mounted significant responses to envelope, a similar number responded to core and only 5/18 (28%) responded to the viral polymerase. Here, envelope appears to be less immunogenic, however, the CD8+ T cell populations in these individuals are constantly exposed to viral antigens, surface antigen in particular. Thus, quantitative differences in antigen load cannot explain the decrease in immunogenicity, but it is more likely that tolerogenic mechanisms begin to over-rule immunogenic potential.

Finally, in the group with HBV DNA $>10^7$ c/ml, none of the 11 subjects that were studied exhibited any response to the core protein but one individual had a response to polymerase. 5/18 patients (28%) however maintained a response to the envelope protein. Again, relative protein quantities cannot account for immunogenicity. These individuals have high levels of circulating virus, as well as envelope subviral particles and e antigen. Polymerase is by far the least available protein. It is apparent in this group that the core protein is highly tolerogenic as it has eradicated this response. Envelope is also highly tolerogenic but not exclusively - a small proportion of these patients maintain responses. The presence of polymerase-specific CD8+ T cell is perplexing. It is possible that an escape mutation could have arisen thereby allowing persistence of the originally primed population but this patient needs to be investigated further. Previous studies have indicated that polymerase is less tolerogenic than envelope (Kakimi et al., 2002) but a response was only seen one patient, therefore, it appears that the relatively limited quantity of this non-structural protein may affect its immunogenicity.

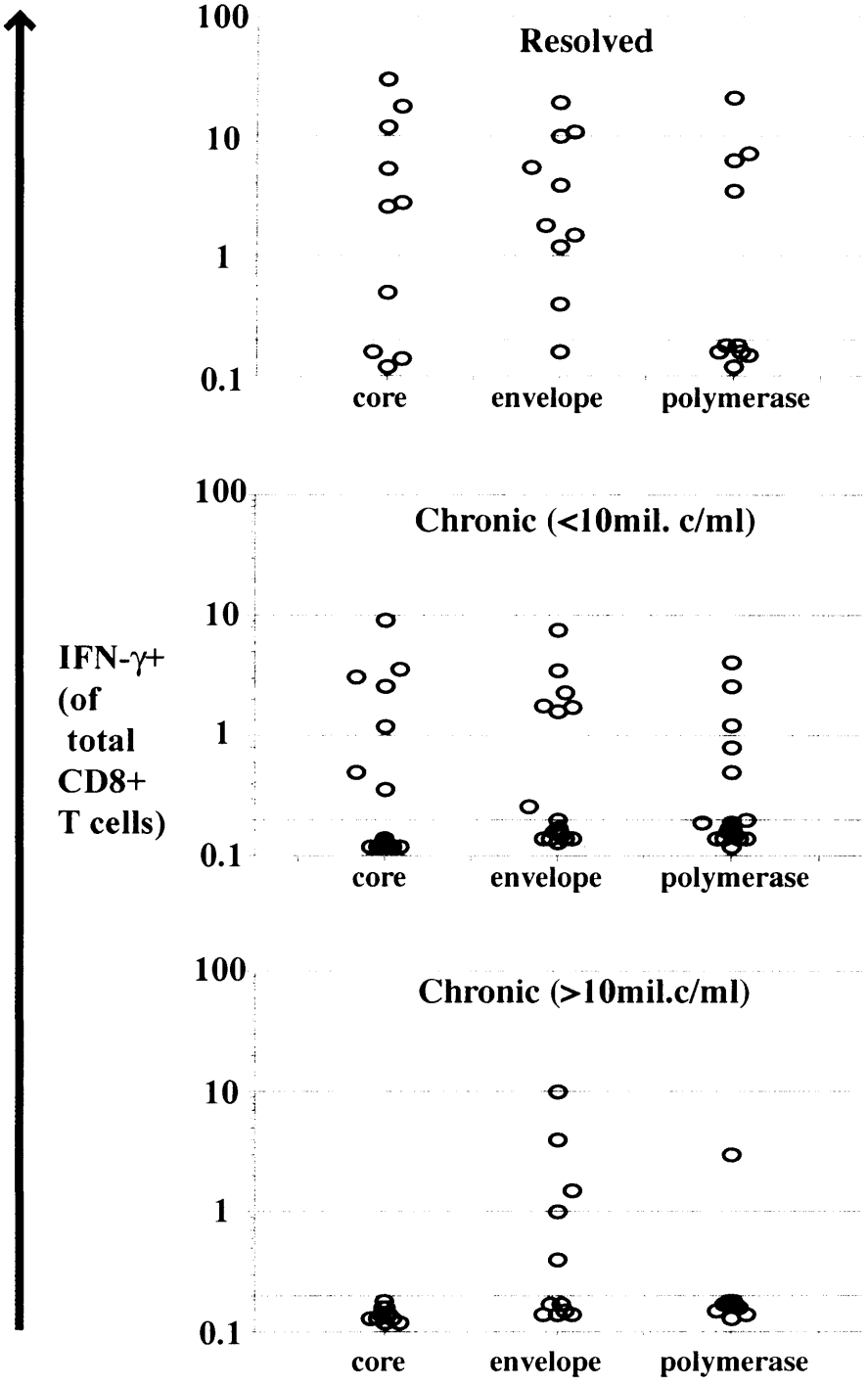

Figure 3.9
HBV-proteins targeted by antiviral CD8+ T cells
The plots indicate the cumulative frequencies of core-, envelope- and polymerase-specific CD8+ T cell responses that were detected in short-term lines. Patients were clustered into one of three groups: resolved (HBV DNA negative), chronic low DNA (<10 million copies/ml) and chronic high DNA (>10 million copies/ml.

3.2.7 Progressive loss of core- but not envelope-specific CD8+ T cell responses occurs with an increasing viral load

To conclude this analysis, we investigated whether there was a relationship between the strength of response of the most immunogenic determinants that were identified in the resolved individuals (core 18-27 and envelope 183-191) and progressive loss of viral control.

This part of the study was performed in close collaboration with G.Webster *et al.*, (U.C.L) who were conducting a parallel longitudinal analysis of HBV-specific CD8+ T in patients with varying outcome that involved a similar 10 day screening of PBMCs from patients for core 18-27 and envelope 183-191 responses (Webster et al., 2004). These data were incorporated in to our results in order to increase the patient numbers within the individual groups of this analysis.

We observed a statistically significant difference in the median frequency of the core 18-27-specific CD8+ T cell response between the resolved and the HBV DNA $<10^7$ copies/ml group ($p<0.0001$). This was also evident between the HBV DNA $<10^7$ copies/ml and the HBV DNA $>10^7$ copies/ml group ($p=0.001$) (figure 3.10 – top) and suggested that this specificity was specifically being silenced by some factor associated with an increase in antigen load. By contrast, the frequencies of envelope 183-191-specific CD8+ T cells did not exhibit a significant stepwise reduction with increasing viral load as observed for core 18-27 (figure 3.10 – bottom).

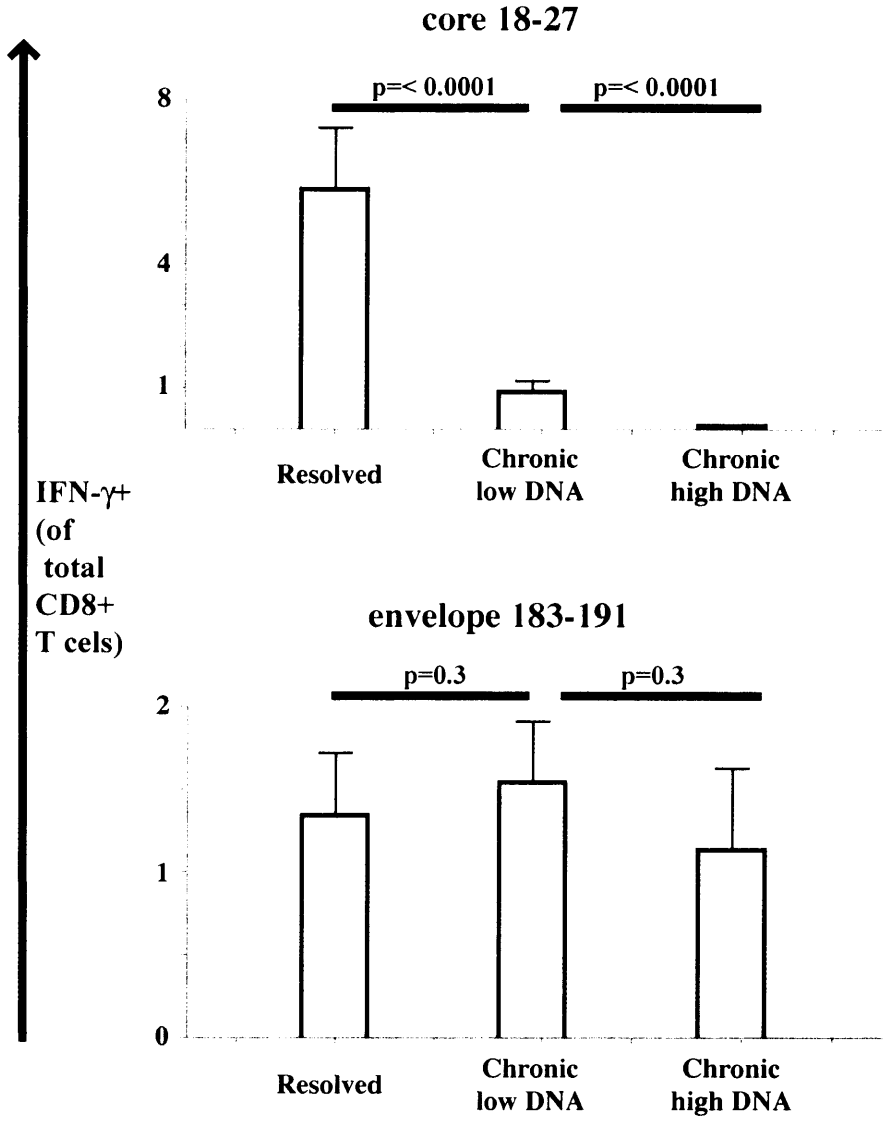

Figure 3.10
Increasing viral load associated with loss of core- but not envelope-specific CD8+ T cell responses
The mean (with standard error) frequencies of core 18-27- (a) and envelope 183-92-specific (b) CD8+ T cells detected after short-term culture are plotted. Patients were groups into the three categories: [(resolved (undetectable DNA), chronic low DNA (<10million copies/ml) and chronic high DNA (>10 million copies/ml)]. The statistical significance of differences between categories (gaussian approximated two-tailed Mann-Whitney test) for the core and envelope responses is indicated above the plots.

3.3 Discussion

The peripheral circulation of resolved individuals contains very low frequencies of HBV-specific CD8+ T cells and these are much lower (if not undetectable altogether) in individuals with chronic infection. In the former group, these cells are most likely to be transient populations sampled as they transit between secondary lymphoid organs. In this way they are able to carry-out systemic surveillance to better respond to a secondary infection or a resurgence of residual virus contained but not cleared after the primary infection. In chronic infection, the antigen-specific cells that can be detected in the peripheral circulation may be those recently released from draining lymph nodes following priming, or those that have escaped arrest at the liver.

The CD8+ T cell compartment comprises approximately 20% of the total mononuclear cell population. This meant that a direct-*ex vivo* antigen-specific population of 0.5% (of total CD8+ T) amounted to one antigen-specific CD8+ T cell per 1000 total PBMCs. Therefore, to confidently determine the frequency of antiviral CD8+ T cells by flow cytometry in any given PBMC sample, a very large number of (stained) cells had to be acquired; ~100,000 total cells to detect 100 virus-specific cells.

However, these data are difficult to interpret objectively because of the variable background false-positivity that arises due to fluorescence resonance energy transfer or non-specific binding; this increases proportionally in relation to the quantity of cells acquired. These factors significantly complicated the evaluation of low-frequency populations - in our hands the typical background amounted to 0.2% of total CD8+ T cells (effectively 40 of 100,000 total flow cytometric events acquired).

Recently, a technique involving magnetic enrichment of multimer-labelled cells has been reported that enables the detection of specific antiviral CD8+ T cells that normally fall below the sensitivity of a direct analysis (Barnes et al., 2004). Although this technique cannot be applied to virus-specific CD8+ T cells that do not bind multimers, this could be over come by adapting the technology so that selection is

performed on the basis of effector function such as IFN-γ production/secretion or degranulation (CD107a/b), however, the major limitaton is the requirement for a relatively large quantity of biological material.

For these reasons, we decided to execute our study by employing a short-term (10 day) *in vitro* virus-specific CD8+ T cell enrichment procedure (previously optimized by our group). A single round of stimulation with HBV peptide followed by culture was used to drive activation-induced proliferation of the CD8+ T cells capable of recognising and responding to the specific stimulus. Supplementation of the growth medium with IL-2 on day 4 sustained the dividing cells up to the tenth day of culture. On day 10 a second peptide stimulation initiated IFN-γ production in the expanded antigen-responsive cells; these were then quantitated by flow cytometry following appropriate antibody staining.

The most effective technology that should have been used for this study would have been the ELISPOT assay in conjunction with overlapping peptides (Tobery et al., 2001). Apart from being comparatively less-labour intensive, ELISPOT assays are capable of very high sensitivity (1/50,000 of total CD8+ T versus 1/2000 by ICS (Yewdell, 2006)). Moreover, as it is not HLA-restricted, it offers an unbiased global screen for all possible CD8+ T reactivity. By starting off with several pools of peptides, one is able to focus-in and to delineate individual viral determinants, which can then also be reconfirmed by ICS. In this way, the investigator can generate a profile of the immunodominance hierarchy of the individual under study that is unrivalled by the competing techniques. The major limitation is the difficulty of utilizing peptides corresponding to the specific viral genotype involved.

We decided to focus specifically on HLA-A2 restricted individuals. The peptide binding motifs for these molecules were the first to be discovered and as a result the immunogenicities of all HBV determinants with the potential to bind HLA-A2 complexes were characterised (Falk et al., 1992; Ferrari et al., 1992; Hobohm and Meyerhans, 1993; Lim et al., 1996; Rotzschke et al., 1992). A large panel of those

previously found to be immunogenic and therefore likely to be targeted by other HLA-A2+ individuals were selected and employed in our screening assay.

The results from this work generated with up-to-date assays are consistent with the findings from earlier studies that demonstrate that the HBV-specific CD8+ T cell response in resolved infection is quantitatively more robust and qualitatively more diverse in individuals that have resolved infection compared to those that maintain chronicity (Bertoni et al., 1997; Nayersina et al., 1993; Rehermann et al., 1995a; Rehermann et al., 1995b).

HBV does not exhibit high rates of mutation during chronic infection (Rehermann et al., 1995b) unlike retroviruses such as HIV and RNA viruses such as HCV thus polyclonality (at the level of individual specificities as demonstrated for HIV (Lopes et al., 2003)) would not appear to be a quality that contributes particularly critical antiviral function. We did however find that HBV core-specific CD8+ T cells were able to tolerate mutated determinants during the persistent phase of the infection (Maini et al., 2000b) and that distinct CD8+ T cells could be primed to natural variants of HBV (Riedl et al., 2006), therefore we cannot eliminate a role for polyclonality.

Our data however, supports the theory that the ability to contain this virus is related to a strong CD8+ T cell response that targets multiple viral components (Bertoni et al., 1997; Chisari and Ferrari, 1995; Rehermann et al., 1995a). This study was focused specifically on the HLA-A2 restricted response, and does not account for the overall antiviral immunity present. The inclusion of the HLA-B and HLA-C would potentially increase the quantity of viral determinants that could be displayed and this could be increased even further by heterozygosity at these alleles. Although studies have shown that a greater allelic diversity associates with better viral control (Nelson et al., 1997), particularly with rapidly mutating pathogens, HBV is a very small virus and is very efficiently organized. It contains only four open reading frames that overlap each other. Despite the involvement of a reverse transcriptase during viral replication (that can contribute to an increased rate of mutation) there are likely to be

significant structural and functional constraints that limit protein modification and thus viral change. This imposes a ridigity and consistency of viral determinants that are presented by the hosts MHCI, but there are rare exceptions (Bertoletti et al., 1994). In this case, mutations could have been forced by certain underlying factors, perhaps HLA homozygosity, which could place acute pressure at a particular determinant forcing the development of the mutant thereby allowing persistence of the modified virus.

Nonetheless, acute infections are normally associated with multi-specific CD8+ T cell responses thereby making it extremely difficult for the virus to escape through multiple simultaneous morphological adaptations, particularly in heterozygous individuals where even more viral targets may be available. Therefore, rather than survive by escaping detection it is highly likely that HBV utilizes survival mechanisms exerted through overt presentation of viral antigens, also termed high zone tolerance, as has been demonstrated in LCMV infection (Moskophidis et al., 1993).

The majority of individuals in this study with effective viral control exhibited vigorous multi-specific CD8+ T cell responses that in addition to limiting viral escape through mutation would have conferred a better capacity to more rapidly contain and purge the pathogen before it was able to stably colonize the host and expedite survival mechanisms to enable its persistence. This is critical in view of the propensity of CD8+ T cells to exhaustion following prolonged or excessive antigenic stimulation (Moskophidis et al., 1993; Wherry et al., 2003).

Polymerase-specific CD8+ T cells were only detected in a minority of the resolved individuals that we tested, suggesting that this specificity, although capable of achieving immunodominance, did not normally occupy a prominent position in the overall antiviral immune hierarchy. The infrequency of these populations in the resolved group did not permit a relative assessment with increasing viral load; only 20% of the resolved sample and 13% of the chronic carriers responded to polymerase. We did however observe polymerase-specific CD8+ T cells in one individual with

high viral load but we were not able to assess the tetramer-binding capacity of these cells at the time; this could have permitted escape from deletion as has been observed and implicated for envelope-specific CD8+ T persistence (Reignat et al., 2002). Alternatively, a mutation in the viral determinant could have allowed persistence of populations primed with the original determinant, as demonstrated in two patients infected with HBV with an altered core 18-27 determinant that maintained CD8+ T specific for the wild-type determinant (Bertoletti et al., 1994).

Unlike core and surface antigen, the polymerase protein is expressed at a much lower quantity. There is no described secreted form apart from that contained in infectious viral particles. Each virus particle contains greater than 100 envelope proteins for every polymerase polypeptide and core proteins that form the nucleocapsid also occur in greater excess compared to the polymerase (Kakimi et al., 2002). It is very likely that peptides from HBe, core and envelope antigen could be preferentially saturating the MHCI presenting complexes on the pAPC surface thus biasing the priming towards HBe/core antigen and envelope specificities rather than to polymerase. This is not exclusively the case in all individuals; polymerase peptides do have high immunogenic potential as observed in two resolved patients that harboured this response. It was found to be stronger than envelope183-191 and even dominated core18 in one individual. Nonetheless, it is unlikely that polymerase specificities are subject to overt peripheral or central tolerance as we propose is occurring in chronic infection with core and envelope specificities.

It is more probable that the low incidence of these populations could be due to competition of determinants for MHCI binding following intracellular processing that then extends to competition between different CD8+ T cell specificities for T cell priming by the pAPCs. Alternatively, suboptimal functional avidity could be responsible; polymerase-specific CD8+ T cells exhibited 20 fold lower differences in effector function compared to envelope-specific CD8+ T cells (Kakimi et al., 2002). However, one needs to take account that these results were based on murine studies comparing two CD8+ T clones generated after multiple rounds of peptide stimulation and prolonged *in vitro* culture. A third possibility could simply be down to the

random nature of antigen encounter that would be influenced by differences in the T cell repertoire exiting the thymus (Attuil et al., 2000); immunodominance would in this instance be attained by specificities that were present at higher precursor frequencies.

Among the other responses detected in the resolved group, core 18-27-specific CD8+ T cells were frequently observed consistent with several studies that demonstrated a strong association of this specificity with self-limited infection (Bertoletti et al., 1993; Bertoletti et al., 1991; Bertoletti et al., 1997b; Maini et al., 1999; Webster et al., 2004).

This has important significance with regard to the initial CD8+ T cell priming event as well as the immuonogenicity of this particular viral determinant: Although naïve CD8+ T cells can be primed at the site of infection (Bertolino et al., 2001; Bertolino et al., 1998), this is unlikely to be a frequent event; the majority of naïve CD8+ T cells reside in secondary lymphoid organs and according to current dogma priming occurs in local draining lymph nodes not at the surface of the hepatocyte. Antigen presenting cells within the liver such as Ito and Kuppfer cells have been implicated in T cell priming (Unanue, 2007; Winau et al., 2007) but the impact on viral infection has yet to be demonstrated. The infection of pAPCs by HBV is not well supported (Untergasser et al., 2006), therefore in order to prime core 18-27-specific CD8+ T cells, pAPCs must be able to capture, efficiently process and finally cross-present core protein that is most likely derived from whole viral particles or circulating e antigen. Given the relatively high quantity of surface antigen that is also present, the dominance of the core18 determinant suggests either a stronger and more stable affinity to the MHCI complex, the presence of a higher naïve precursor frequency of CD8+ T cells specific for this peptide or alternatively, that the functional avidity of c18-27 specific precursors may be greater than those specific for alternative viral targets. It is possible that more than one of these factors could have occurred simultaneously.

The implication of the overall dominance of core18 in the majority of resolved individuals is that this determinant bears some physiological advantage that allows it

to out-compete others and this highlights its immunogenic superiority and relevance to effective viral control. However, although this may be the case in resolved infection our data indicates that there is a particularly strong inverse association of this specificity with increasing viral load. This may be because the antiviral CD8+ T cells are being sequestered in the liver, however, viral load was found to be inversely proportional to the frequency of intrahepatic core 18-27-specific CD8+ T cells (Maini et al., 2000a; Reignat et al., 2002). Otherwise, viral mutants may be implicated; although we have not sequenced the viral genomes in our study sample it is unlikely that a core variant has occurred in all the chronic individuals that exhibited the loss of this specificity. This is supported by another study with a large number of chronically infected patients associated with loss of core18-specific CD8+ T cells that did not reveal any core variants (Reignat et al., 2002).

The most plausible explanation for the loss of core 18-27-specific CD8+ T cells in chronic infection may simply be due to the fact that they are gradually being overwhelmed by persistent antigenic encounter and driven to exhaustion as has been reported previously (Fuller and Zajac, 2003; Moskophidis et al., 1993; Tewari et al., 2004). High levels of viral replication is associated with the secretion of large quantities of virus but also of e antigen (the modified version of core), a protein that has been reported to be more efficient at eliciting T cell tolerance than HBcAg (Chen et al., 2005). This by-product of viral replication, as well as surface antigen and whole virions, has the potential to exert anti-immune function through multiple mechanisms:

1) Professional APCs are capable of capturing, processing and presenting the e antigen and preferentially priming naïve T cells specific for the core 18-27 determinant. In this way early specificities may be more prone to exhaustion following continuous encounter with antigen, as has been observed in other persistent viral infections (Fuller and Zajac, 2003; Probst et al., 2003; Wherry et al., 2003).

2) Studies in transgenic mice models of persistent HBV infection have demonstrated that the tolerogenic activity of e antigen could be mediated by driving the T helper

response to a type two profile (Milich, 1997; Milich et al., 1998) typically characterised by the production of IL-10. This cytokine can suppress antigen-specific CD8+ T responses (Abel et al., 2006; Elrefaei et al., 2007) and may well be suppressing core 18-27-specific CD8+ T cells.

3) pAPCs capture circulating antigen and migrate to the thymus thereby inducing central tolerance (Bonasio et al., 2006). Interestingly, not all "self-reactive" CD8+ T cells are deleted; some antigen-experienced specificities exit and circulate among the peripheral compartment but are functionally silenced; this is more relevant to envelope-specific CD8+ T cells and is addressed in the subsequent section.

4) The modulation of surface CD8 expression can dramatically influence the effector response (Maile et al., 2005). High avidity CD8+ T cells can overcome this but sub-optimal responses are generated by low avidity populations. Although some evidence suggests that core 18-27-specific CD8+ T cells are high-avidity populations, these data are derived from assays utilizing extensively cultured T cell clones; further studies into the expression and interaction of this co-receptor are necessary.

5) Cross presentation of antigen by APCs has been shown to mediate peripheral CD8+ T cell tolerance (Belz et al., 2002a; Belz et al., 2002b; Belz et al., 2002c; Davey et al., 2002; van Endert and Villadangos, 2007; Villadangos, 2007; Villadangos et al., 2007). This could be because antigen capture without appropriate inflammatory danger stimuli may not initiate appropriate maturation (Anderson et al., 2001; Gallucci and Matzinger, 2001; Matzinger, 2002). HBV is a non-cytopathic virus and chronic infection can be maintained for very long periods without any obvious liver damage (as determined by the level of serum ALT). Uptake of HBV antigens in the absence of inflammatory signals may only result in suboptimal maturation of the pAPCs and the subsequent priming of core 18-27-specific CD8+ T cells by these APCs could potentially initiate apoptosis leading to deletion. Antigen specific B cells able to function as antigen presenting cells have also been implicated in this mechanism (Raimondi et al., 2006a, b).

These factors acting either independently at different phases of the infection or in unison could account for the loss of the core 18-27-specific response during viral persistence. Further work is necessary to identify and understand better the precise mechanism that influences this critical CD8+ T cell specificity and tips the balance from effective viral containment towards chronic and uncontrolled viral replication.
Envelope 183-191-specific CD8+ T cell responses were, following core 18-27-specific CD8+ T cells, the next most vigorous and frequent T cell population that we detected in the resolved individuals indicating that this determinant also had good immunogenic potential and could be contributing important antiviral function necessary to achieve viral control. The reduction in the overall robustness of this response in chronic infection suggested either a proliferative impairment or a propensity to exhaustion similar to that seen for core 18-27-specific CD8+ T cells.

Importantly, envelope-specific CD8+ T cells still persisted in several individuals despite extremely high levels of circulating antigen. Although persistent infection is associated with the secretion of subviral particles composed of envelope protein at quantities of 1-300μg/ml - 10^3 to 10^6 fold in excess over whole virions (Kim and Tilles, 1973), it was striking that these specificities did not exhibit a reduction with increasing viral load as was observed with core18-27-specific CD8+ T cells. This phenomenon of CD8+ T cell escape has been reported previously but is not properly understood. It is not unique to HBV, but may be the outcome of some as yet unidentified common mechanism allowing certain antigen-specific CD8+ T cell to escape high antigen load exhaustion (Spencer and Braciale, 2000). A mutation in the determinant could have explained persistence (akin to the core 18-27 mutation) – the inability to engage an altered determinant would rescue the population from deletion. This could also account for the inability to exert significant immune pressure in vivo, however, mutations were not found in several individuals in whom the virus was sequenced (Reignat et al., 2002). Additionally, TCR down-modulation was not obvious and neither was lowered peptide avidity - envelope-specific CD8+ T cells from chronic infection exhibit good IFN-γ production, proliferative potential and cytotoxicity when exposed to viral peptides or antigen loaded target cells (Reignat et al., 2002).

It is certainly likely that some of these cells are exhausted perhaps through mechanisms proposed for the deletion of the core 18-27 specificity; this would explain the overall reduction in the strength of this response in chronic infection. The populations that do manage to persist may be the exceptions and the most likely explanation for their survival could be related to their altered phenotype; their inability to bind HLA-matched tetramers suggests defective TCR organization. Incomplete maturation could also be a factor (Spencer and Braciale, 2000) but additional studies into membrane fluidity, lipid raft organization and TCR recruitment are needed to further understand them.

As this response was associated with effective viral control in the absence of the core 18-27 CD8+ T cell population our data indicate that successful immunity can be achieved by lesser-dominant specificities. However, we cannot overlook the fact that a CD8+ T cell population specific for a viral determinant not included in our peptide panel could be contributing the antiviral activity responsible for the effective viral suppression. This would have been clarified if we had screened for CD8+ T cell reactivity with overlapping peptides covering all HBV proteins.

Nonetheless, these findings are very relevant to patients with chronic infection where the immunodominant core response has been deleted but other specificities remain, principally because it presents an opportunity to return effective antiviral functionality to persisting populations; this is also an encouraging prospect given that subdominant responses have previously been implicated in the clearance of chronic infection (Frahm et al., 2006; van der Most et al., 1998; van der Most et al., 1996).

Unlike expansion and contraction that is programmed during the initial priming (Iezzi et al., 1998; Kaech and Ahmed, 2001; van Stipdonk et al., 2001), T cell function is modulated at later stages, and functional T cell inactivation that occurs early in persistent infection is reversible and can be altered by reprogramming (Brooks et al., 2006a). This was achieved by removing the cells from the antigenic environment and also by artificially reducing the antigenic load through the administration of antiviral

drugs. These authors also suggested that an extended period of inactivation resulted in the accumulation of intrinsic functional defects. A better understanding of the precise nature of this dysfunction would help address better the mode of correction that would be required.

In this study the hierarchies of various antiviral-CD8+ T cell populations in resolved and chronic infection have been evaluated using selected HLA-A2 restricted viral peptide determinants and although we observed that hierarchical trends were similar to the respective proteins, it is not entirely possible to determine whether the differential immunogenicity is due to the protein derivation: core, envelope or polymerase.

The inability of HBV to infect pAPC (Untergasser et al., 2006) suggests that differential protein expression kinetics would not influence the immunogenic hierarchy that is generated as has been reported for other viruses (Hislop et al., 2002). Although polymerase is one of the earliest HBV proteins to be synthesized, this occurs within the infected hepatocyte and as infection is not cytopathic there should not be any release of polymerase protein into the circulation until the initiation of the innate immune response; the exception would be circulating virions, however core and envelope proteins make up a much larger proportion of the total.

In the interim, prior to the NK activity that we have recently shown to be responsible for the initial liver damage following the immunotolerant phase (Dunn et al., 2007), it is most likely that peripheral dendritic cells that have encountered and taken-up soluble HBV products such as subviral particles, e antigen and whole virions, would have migrated to local draining lymph nodes. At that location there would be a preferential exhibition of determinants derived from core and envelope proteins favouring the observation of a preferential immunodominance by core and envelope-specific CD8+ T cells in resolved individuals. These immunodominant specificities may suppress subdominant ones, especially when the antigen is limited (Hislop et al., 2002). Persistent antigenic stimulation due to the inability to effectively reduce viral replication is likely to gradually erode the primary immunodominant specificities

such as core 18-27- and envelope-183-191-specific CD8+ T cells unless they either naturally possess or adapt mechanisms for survival. Data from a transgenic mouse study indicate that the HBV envelope protein but not the polymerase is tolerogenic and the authors suggest that the cause for this is a relatively lower level of expression coupled to low functional avidity for the antigen (Kakimi et al., 2002).

This raises another issue: intrinsic properties of individual determinants including efficiency of presentation and MHCI binding affinity are also key factors very likely to govern immune hierarchies. Several studies have indicated that the abundance and stability of viral peptide presented by the host MHC correlates with the magnitude of the virus specific CD8+ T cell response (Busch and Pamer, 1998; Gallimore et al., 1998c; Pion et al., 1999). Furthermore, protective immunity has been reported to be a function of the affinity of the interaction between the individual CD8+ T cell and the target cell (Anton et al., 1997; Gallimore et al., 1998a; Gallimore et al., 1998b; Gallimore et al., 1998c; Restifo et al., 1995; Tsomides et al., 1994) with importance placed on the ability of the antiviral cells to detect low concentrations of viral peptide. In contradiction with these findings a study of EBV infection has suggested that immunodominance may not necessarily correlate with viral peptide abundance (Crotzer et al., 2000), however, the authors have acknowledged that chronic stimulation by abundant determinants could have resulted in the deletion of certain specificities. Nonetheless, apart from peptide abundance the study raises the possibility that alternative factors may influence immunodominance.

An additional factor that is poorly understood but extremely relevant to the priming of CD8+ T cells is the precursor T cell repertoire. The generation of a specific CD8+ T cell response relies on the presence of naïve CD8+ T cells that are able to recognize the presented viral peptide. "Holes" in the repertoire make abundant peptides redundant. Additionally, abundant but ineffective peptides have the potential of saturating MHCI complexes leaving little room for alternative peptides that do have complementary CD8+ T cell populations. Thus, the precursor frequency is a critical issue that merits further investigation; it is a very difficult factor to study in humans but may be better illuminated through murine studies (La Gruta et al., 2006).

The precursor frequency in turn highlights the importance of heterologous immunity. A large proportion of the studies into immunodominance hierarchies have been performed in murine models. While mice are maintained in extremely clean environments, humans are constantly exposed to numerous pathogens that steadily mould their T cell memory repertoire. A cross-reactive memory response is mounted faster than one primed *de novo* due to a large precursor frequency and the inherent ability of these differentiated populations to respond faster (Selin and Welsh, 2004). The difference in the response rate may theoretically provide a critical advantage that could account for better control (Cornberg et al., 2007), however, the opposite is also possible. Heterologous viral challenge in a murine study resulted in the generation of a relatively restricted and suboptimal repertoire that was associated with viral escape (Cornberg et al., 2006). Additionally, acute HCV infection resulted in more severe hepatitis in individuals that exhibited HCV-specific CD8+ T with cross-reactivity to an influenza protein (Urbani et al., 2005). Ultimately, the private repertoire of each individual human, as influenced by their previous history of infection, influences the CD8+ T cell hierarchy and thus efficacy of subsequent responses. It is important to acknowledge that although much has been gained, there are fundamental differences between murine studies and humans. Heterologous immunity is a major concern, but other important factors including the strain of virus involved, the dose of the inoculant, and the route of infection also need to be considered (Tscharke et al., 2005); these are normally standardized in murine studies and the implications have only recently begun to be appreciated in humans.

In conclusion, these findings have important implications for the selection of determinants in the design of vaccines aimed at inducing CD8+ T cell responses in chronic infection. According to our data on a limited number of HLA-A2-restricted HBV determinants, core 18-27- and envelope 183-191-specific CD8+ T cells are highly prevalent in HLA-A2+ individuals that exhibit effective viral control. This is most likely due to the abundance of these proteins, suitability for efficient processing and the availability of a precursor repertoire during the initial stages of infection. However, it appears that these very factors make these populations especially prone to exhaustion if viral replication is not effectively reduced within a certain critical period. In order to optimize their antiviral efficacy it is imperative that the pathogen is reduced to a quantity that is sufficient to initiate but not overwhelm the effector response. This is feasible through antiviral drug therapy and is likely to be more effective at an early stage of infection when the primary immunodominant responses have not yet been deleted. In the event that this has already occurred, alternative specificities that were not initially successfully primed could be specifically targeted. Lastly, it is still important to understand better the precise nature of CD8+ T cell dysfunction that is associated with increasing antigenic load that could also help address alternative persistent viruses such as HIV and HCV. The rare populations of envelope-specific CD8+ T cells present in chronic infection offered the opportunity to further investigate these mechanisms; these studies are the focus the following two chapters.

4 CHAPTER 4

Application of cDNA microarrays to the study of HBV-specific CD8+ T cells.

4.1 Background

Chronic viral infection can cause a complex variety of defects in the virus-specific CD8+ T cell population. These include impaired proliferation as well as defects in effector function such as sub-optimal cytotoxicity and cytokine production, aberrations that have been described in several persistent infections namely LCMV, HIV, SIV, HTLV-1 (Appay et al., 2000; Lim et al., 2000; Xiong et al., 2001; Zajac et al., 1998). In the previous chapter we demonstrated that this dysfunction can also occur in persistent HBV infection. We found that this compartment was markedly contracted in a large number of individuals in contrast to a relatively more diverse and robust response in individuals that managed to resolve infection. These data, supported by the recent work of Thimme et al., indicated that the collapse of the virus-specific CD8+ T cell compartment was likely to be a major determinant of persistence of this important human pathogen, leading us to investigate the mechanism driving the paucity of the HBV-specific response in chronic HBV infection.

Although conventional flow cytometry-based phenotyping and functional assays can provide highly accurate information on the sample under study, the major limitations of the technology are the requirement for a relatively large number of cells and restrictions in the number of variables (Pantaleo and Harari, 2006) that can be studied simultaneously. The latter is generally confined to a few parameters that are pre-determined, therefore rendering it an inappropriate methodology for broad surveillance-type investigations. cDNA microarray analysis on the other hand is an alternative technology that provides the ability to conduct a simultaneous assessment of several hundred to thousands of cellular gene transcripts. Importantly, this information can be derived from a very limited number of cells.

Lymphocyte responses that follow a specific stimulus through a surface receptor are exerted through specific proteins. With the exception of those that are stored and ready for immediate function either through secretion or activation (e.g. phosphorylation or methylation) other proteins are generated following de novo transcription from the cellular genome. Transcriptional fluctuations that follow specific triggers can therefore be used to predict protein changes related to a cellular response. A comparative analysis of the cellular transcriptome of independent populations can potentially reveal essential differences, provided that these fall within the sensitivity of the assay. Significantly up- or down-regulated genes can subsequently be selected for focused investigations into biological involvement.

Thus, in order to broaden the scope of our investigation, we decided to complement existing flow cytometry-based assays with a functional genomics approach, allowing us to conduct an extensive unbiased comparative study. The responses detected in resolved individuals provided the functional gold-standard against which ineffective responses associated with chronic infection were compared.

In order to maximize the probability of identifying relevant genes associated with viral persistence it was necessary to profile virus-specific CD8+ T cells at the peak of their dysfunction. HBV-specific CD8+ T cells from individuals with chronic infection with low viral load did not truly represent dysfunctional cells; these populations appear to exert partial antiviral function as is reflected by a low level of viral replication. Rather, classically dysfunctional CD8+ T cell populations are those found in people with high levels of viral replication but these individuals do not normally maintain the typical antiviral responses observed in resolved infection, neither directly *ex vivo* nor following short-term culture. However, we were able to overcome this obstacle by focusing on rare populations of envelope-specific CD8+ T cells that somehow persisted in a very limited number of individuals with chronic infection with high viral loads (>10million/ml). These populations did not appear to have significant in vivo antiviral capacity, as reflected by the excessive quantity of virus and viral antigens present in the serum of these patients.

The application of cDNA microarrays to the investigation of human virus-specific CD8+ T had not been attempted previously and therefore the aim of this work was to adapt and optimize existing protocols to be able to study limited numbers of activated HBV-specific CD8+ T cells thereby enabling us to conduct a comparative analysis of this antiviral immune response in individuals with resolved and persistent HBV infection.

4.2 Results

4.2.1 mRNA Extraction

The assessment of the cellular transcriptome of small numbers of lymphocytes necessitated an appropriate RNA extraction methodology. RNAzol B and Trizol reagent purification methods are based on the guanidium thiocynate phenol method (Chomczynski and Sacchi, 1987) that requires precipitation of the transcripts and additional washes to remove traces of organic compounds that could otherwise interfere with downstream reactions. This method works best with a very large number of cells because significant sample loss can occur (illustrated in figure 4.1). This was not conducive to the purification of RNA from the small numbers of PBMCs (~300,000) that we intended to analyse and so we turned to a Dynal poly-T-linked magnetic beads based approach. The use of this method was supported by its successful application in the heteroduplex analysis where sample limitation was a major problem (Lopes et al., 2003). However, as genearray studies had not previously been conducted with Dynal-purified mRNA it was necessary to determine the suitability and validity of this purification method.

A primary evaluation was conducted on PBMCs taken from an individual that had resolved HBV infection (figure 4.2). 300,000 cells were seeded in quadruplet: one pair of unstimulated and stimulated samples (A-US & A-S) were used to determine the frequency of core-specific CD8+ T cells and the other pair were reserved for mRNA extraction. The samples were enriched for core-specific CD8+ T cells by stimulation with the core 18-27 peptide. At day 10 of culture the cells were pooled and reseeded to ensure that the samples were homogenous; two wells were then restimulated with the core peptide for five hours. Five hours after peptide restimulation, one pair of unstimulated and stimulated samples were stained and analysed by flow cytometry. The second pair of unstimulated and stimulated cells (B-US & B-S respectively) were lysed in 500μl of Dynal lysis solution, the mRNA was extracted, eluted and stored at -70°C.

We selected a stimulation period of five hours because previous studies have indicated that the transcriptional peak of several cytokines, particularly IFN-γ (characteristic of the HBV core-specific CD8+ T cell effector response in resolved infection) was 4 to 6 hours following stimulation (Abdalla et al., 2003). However, the transcriptional levels of certain genes participating in the response could have peaked before or after this time point (Teague et al., 1999) and so the analysis of multiple time points could have provided important information. In order to address this, we also attempted to analyse a time course by comparing the immediate early response (one hour following stimulation), the standard time (5 hours) and the late response (16 hours).

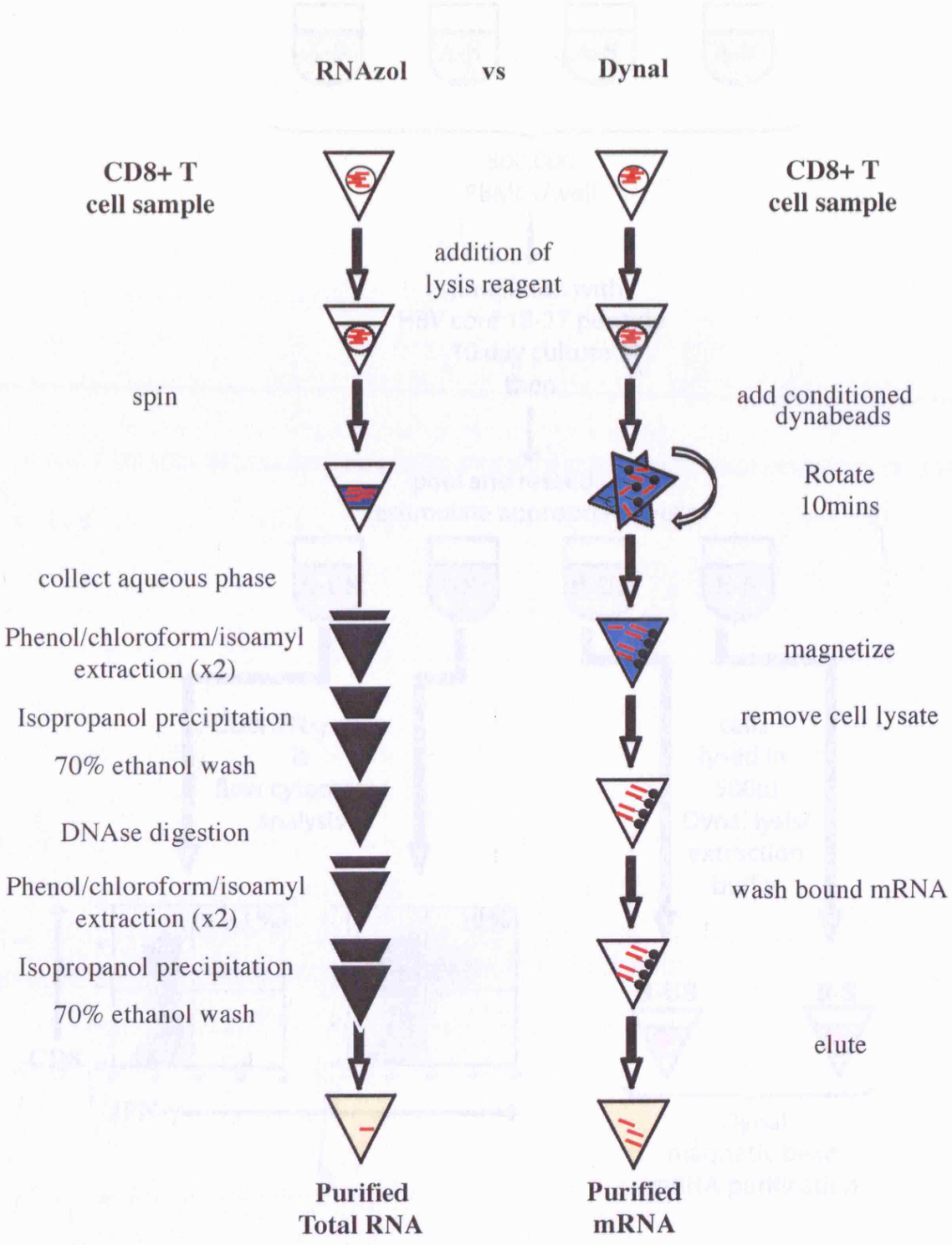

Figure 4.1
Comparison of RNA extraction methods
Schematic illustrating difference between the traditional method of RNA extraction (RNAzol) and Dynabead technology.

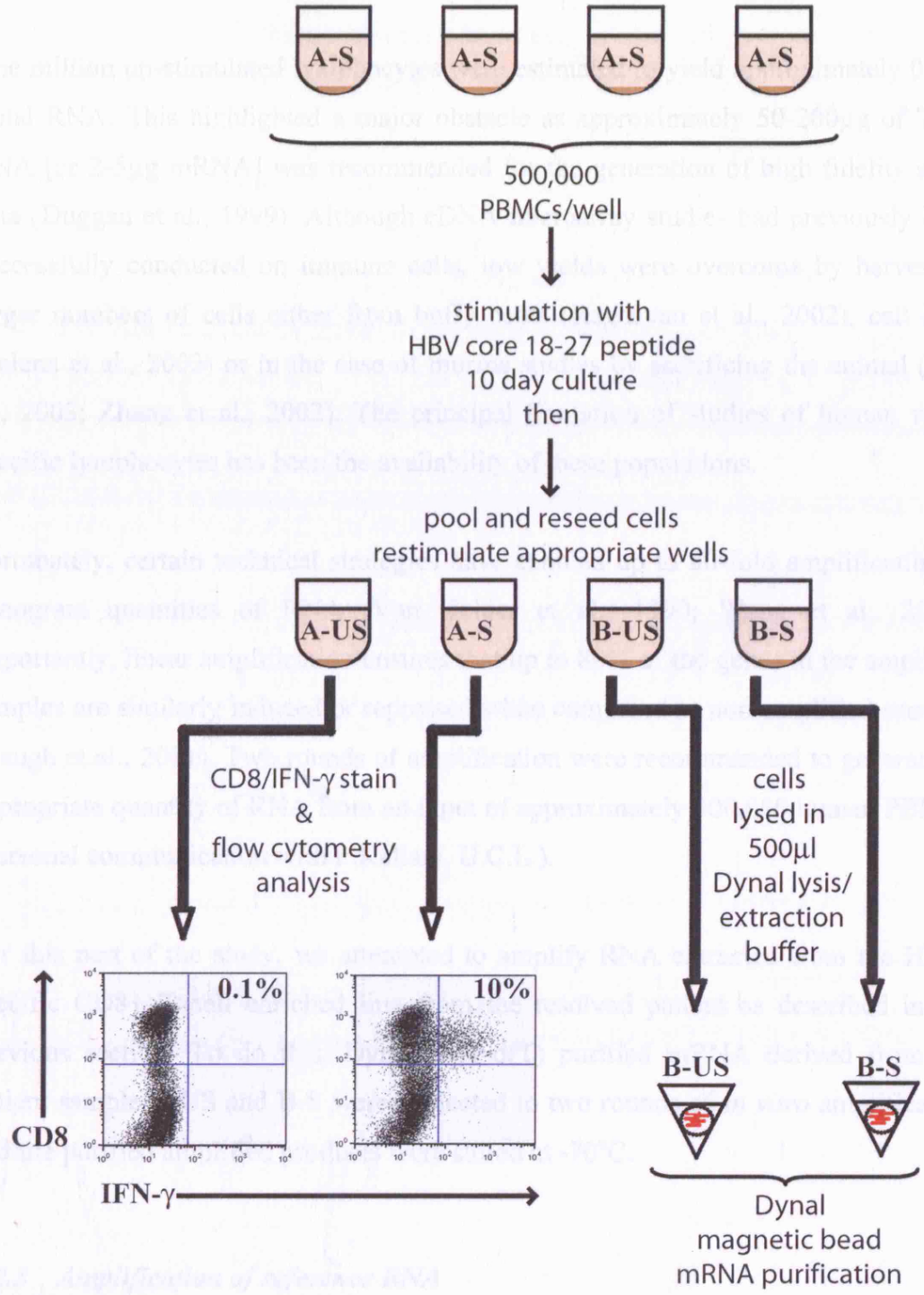

Figure 4.2
Generation of biological samples for cDNA microarray analysis – cell culture to RNA extraction

4.2.2 Amplification of sample RNA

One million un-stimulated lymphocytes were estimated to yield approximately 0.5μg Total RNA. This highlighted a major obstacle as approximately 50-200μg of Total RNA [or 2-5μg mRNA] was recommended for the generation of high fidelity array data (Duggan et al., 1999). Although cDNA microarray studies had previously been successfully conducted on immune cells, low yields were overcome by harvesting larger numbers of cells either from buffy coats (Raghavan et al., 2002), cell lines (Palena et al., 2003) or in the case of murine studies by sacrificing the animal (Ji et al., 2003; Zhang et al., 2002). The principal limitation of studies of human virus-specific lymphocytes has been the availability of these populations.

Fortunately, certain technical strategies have enabled up to 80-fold amplification of nanogram quantities of RNA (Van Gelder et al., 1990; Wang et al., 2000). Importantly, linear amplification ensures that up to 80% of the genes in the amplified samples are similarly induced or repressed when compared to non-amplified samples (Baugh et al., 2001). Two rounds of amplification were recommended to generate an appropriate quantity of RNA from an input of approximately 300,000 human PBMCs (personal communication with P.Kellam, U.C.L.).

For this part of the study, we attempted to amplify RNA extracted from the HBV-specific CD8+ T cell enriched line from the resolved patient as described in the previous section. To do this, Dynal Poly-d(T) purified mRNA derived from the patient sample B-US and B-S were subjected to two rounds of *in vitro* amplification and the purified amplified products were stored at -70°C.

4.2.3 Amplification of reference RNA

Comparative cDNA microarray analysis relied on the incorporation of an equal quantity of artificial reference RNA into every test sample (see section 1.13.2.1). In

order to maintain methodological consistency between the reference RNA and the sample RNA, the reference was also amplified. Additionally, this enabled the production of a large common stock from which identical aliquots could be stored and used when appropriate enriched-cell lines were available.

The first amplification of reference RNA was essentially performed on one unique primary stock of commercially derived total universal reference RNA (TURR), however, three variations of this stock were tested: Test 1 (Ref.A) was conducted using only 1μg total TURR, Test 2 (Ref.B) was conducted on 25μg of TURR and Test 3 (Ref.C) was conducted on mRNA that was purified from 25 μg of TURR. The latter was included to match the RNA extracted from the patient samples. Purified amplified products (aRNA-Ref.A1, aRNA-Ref.B1 and aRNA-Ref.C1) were eluted in RNAse free water, aliquoted, and stored at -70°C.

A second round of amplification of reference was conducted on 5μg of aRNA-Ref.B1 and aRNA-Ref.C1; aRNA-Ref.A1 was omitted because a quantitative preview indicated a poor yield. Highly purified amplification products (aRNA-Ref.B2 and aRNA-Ref.C2) were eluted and stored at -70°C.

4.2.4 Quantification

The efficiency of Cy5- or Cy3-labelling of amplified RNA could be influenced by differences in the quantity of input RNA and it was therefore important to minimize these variations. The traditional means of RNA quantitation such as ethidium bromide stained agarose gels and spectroscopic determination were inappropriate for the quantification of nanogram amounts of aRNA. However, the Agilent Bioanalyser was an instrument that offered highly sensitive determination not only of the quantity of the aRNA samples but also its quality: a smooth uninterrupted RNA profile signified a high quality sample without ribosomal RNA contamination whereas jagged curves indicated degradation.
(see http://www.chem.agilent.com/Scripts/PDS.asp?lPage=51)

In order to determine the quantity of RNA in the first and second round amplified samples B-US and B-S as well as the amplified reference RNA (aRNA-Ref.A1, aRNA-Ref.B1, aRNA-Ref.C1, aRNA-Ref.B2 and aRNA-Ref.C2), a small quantity was loaded onto Agilent Biochips and measured. Additionally, 150ng of ladder (identical to that used in the designated ladder well) was loaded as an internal control.

The left plot in figure 4.3 is the typical mRNA profile of 150ng standard ladder with seven bands (0.2, 0.5, 1.0, 2.0, 4.0 and 6.0kb respectively) that follow the first peak that corresponds to the reporter dye; the 0.2kb band is at a concentration of 20ng/μl. The right plot in figure 4.3 is a profile of the identical ladder that was used as a control to evaluate the fidelity of the quantification. The bioanalyser calculated the internal control sample to contain 158ng of RNA corresponding to <5% error margin.

Figure 4.4 demonstrates the profiles of the amplified resolved samples, where the total yield was 2.37μg and 2.94μg for once amplified and 44.8μg and 41.4μg for twice-amplified samples B-US and B-S respectively; the quantity of the latter pair potentially being sufficient for approximately 8 arrays repeats at 5μg RNA/array. These data confirmed that by coupling Dynabead mRNA purification technology

with a modified linear RNA amplification protocol it was possible to generate sufficient RNA to meet microarray requirements from patient samples composed of only 300,000 PBMCs.

Figure 4.5 (middle horizontal 3 plots) are mRNA profiles for the first round amplified reference RNA (aRNA-Ref.A1, aRNA-Ref.B1 and aRNA-Ref.C1) indicating yields of 624, 3584 and 12731ng/μl equivalent to a total of 25, 142 and 381μg and sufficient for 5, 28 and 76 arrays respectively. The bottom two plots are RNA profiles following the second amplification indicating concentrations of 10.6μg/μl (aRNA-Ref.B2) and 8.2μg/μl (aRNA-Ref.C2) corresponding to final recoveries of 59μgs and 48μgs - sufficient for 9 and 5 arrays respectively.

The best yield of reference RNA obtained was sample aRNA-Ref.C1 (hereafter referred as aREF) that at 5μg per array was ample for at least 70 arrays; this was sufficient for the quantity of samples that we planned to analyse in this phase of the study. Subsequent trials demonstrated that comparably high quality array data could be obtained using only 2μg of amplified RNA thereby doubling the quantity of arrays that could be produced from the remaining aREF stocks.

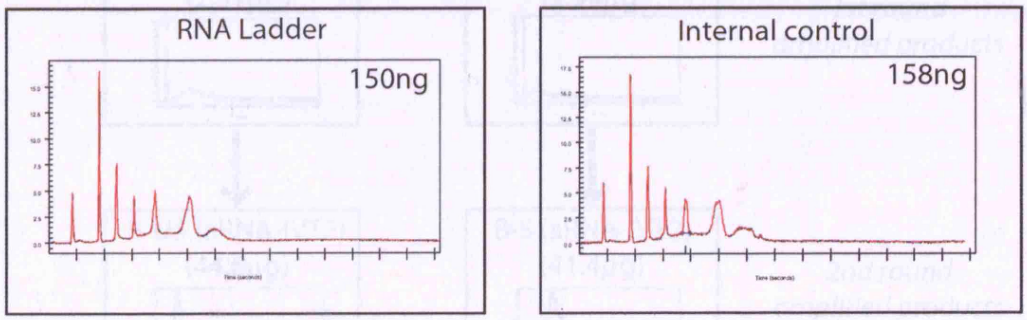

Figure 4.3
RNA Quantification - ladder and internal control
RNA ladder calibration standard (left) and RNA-biochip internal control (right) consisiting of 150ng ambion RNA ladder.

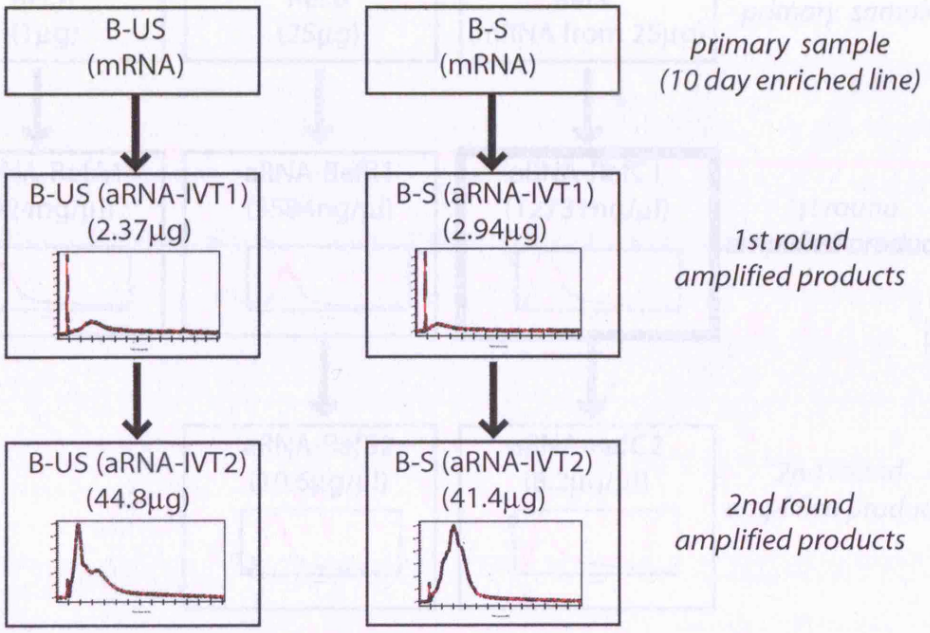

Figure 4.4
RNA quantification - patient sample
aRNA products from *in vitro* amplification (two rounds) of mRNA derived from an unstimulated and stimulated HBV core18-27-enriched line.

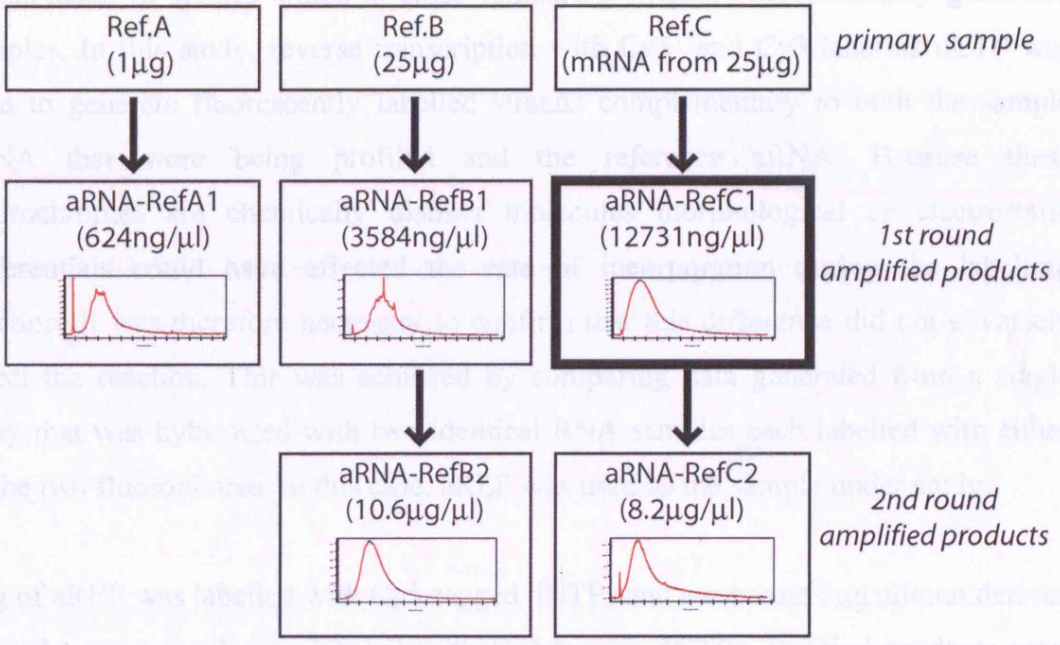

Figure 4.5
RNA quantification - amplified universal reference RNA.
aRNA products from an i*n vitro* amplification (two rounds) of reference RNA from three source stocks (top boxes - left, middle and right). Middle horizontal 3 plots indicate profiles following the 1st round of amplification and the lowest two plots correspond to aRNA profiles following the 2nd round of amplification. The high-lighted plot indicates the sample that was selected as reference RNA for this study (aREF).

4.2.5 Validation of cDNA microarray methodology

4.2.5.1 Dye incorporation (Cy5 vs Cy3)

Dual-colour cDNA arrays allow for a highly effective normalization that is fundamental to studies aimed at cross-comparing multiple independently generated samples. In this study, reverse transcription with Cy5- and Cy3-labelled dCTP was used to generate fluorescently labelled strands complementary to both the sample aRNA that were being profiled and the reference aRNA. Because these fluorochromes are chemically distinct molecules morphological or electrostatic differentials could have affected the rate of incorporation during the labelling reaction. It was therefore necessary to confirm that this difference did not adversely affect the reaction. This was achieved by comparing data generated from a single array that was hybridised with two identical RNA samples each labelled with either of the two fluorophores. In this case, aREF was used as the sample under study.

5μg of aREF was labelled with Cy5-tagged dNTPs and a separate 5μg aliquot derived from the same stock was labeled with Cy3-tagged dNTPs. Purified products were then cohybridized to a cDNA microarray and processed as described in chapter 2. Fluorescent intensities of the hybridized transcripts [for ~5000 individual genes (in duplicate)] were then analysed in excel.

The Cy5 and Cy3 expression data for each spot on the array (~5000 in duplicate) was corrected for local background noise, Log_2 transformed (refer section), and plotted against each other. We found that the gene transcripts labelled with Cy5 generated fluorescent signals that were highly similar to that obtained when labelled with Cy3 (figure 4.6; r=0.92) demonstrating that molecular differences between the fluorochromes did not have any significant negative influence over the labelling reaction and hybridization to the array probes.

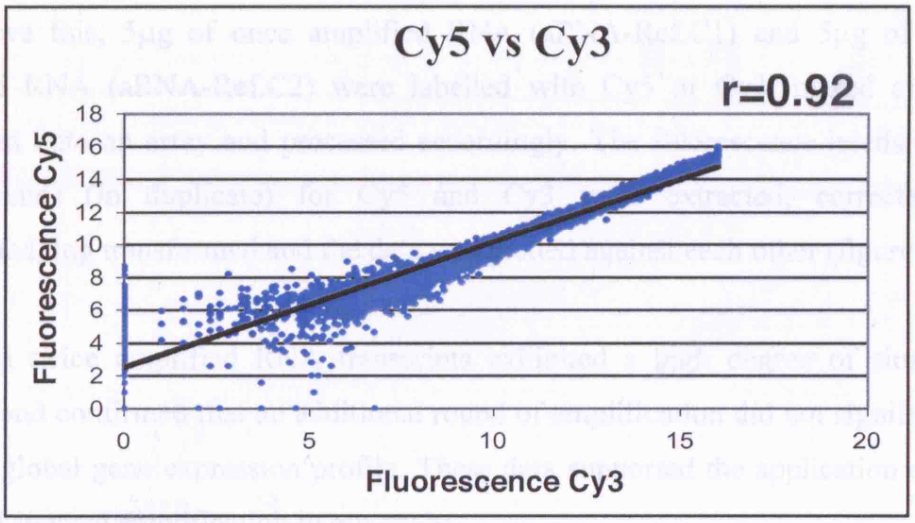

Figure 4.6
Comparative efficiency of Cy5 and Cy3 dye incorporation.
The labelling efficiency of Cy5- an Cy3-labelled dUTP was compared by plotting the fluorescence intensities of a fixed quantity of identical reference RNA simultaneously labelled with either of these dyes and co-hybridized to an HGMP cDNA microarray.

4.2.5.2 Two rounds of amplification

The generation of sufficient aRNA from small numbers PBMCs necessitated two rounds of *in vitro* amplification. Although studies have shown that a single round of amplification does not significantly alter the gene expression profile compared to unamplified samples, we needed to confirm that an additional round of amplification would not compromise the fidelity of the data.

To achieve this, 5µg of once amplified RNA (aRNA-Ref.C1) and 5µg of twice amplified RNA (aRNA-Ref.C2) were labelled with Cy5 or Cy3, mixed and co-hybridized onto an array and processed accordingly. The fluorescence intensities of ~5000 genes (in duplicate) for Cy5 and Cy3 were extracted, corrected for background, log transformed and the data was plotted against each other (figure 4.7).

Once and twice amplified RNA transcripts exhibited a high degree of similarity (r=0.96) and confirmed that an additional round of amplification did not significantly alter the global gene expression profile. These data supported the application of two rounds of *in vitro* amplification in our study.

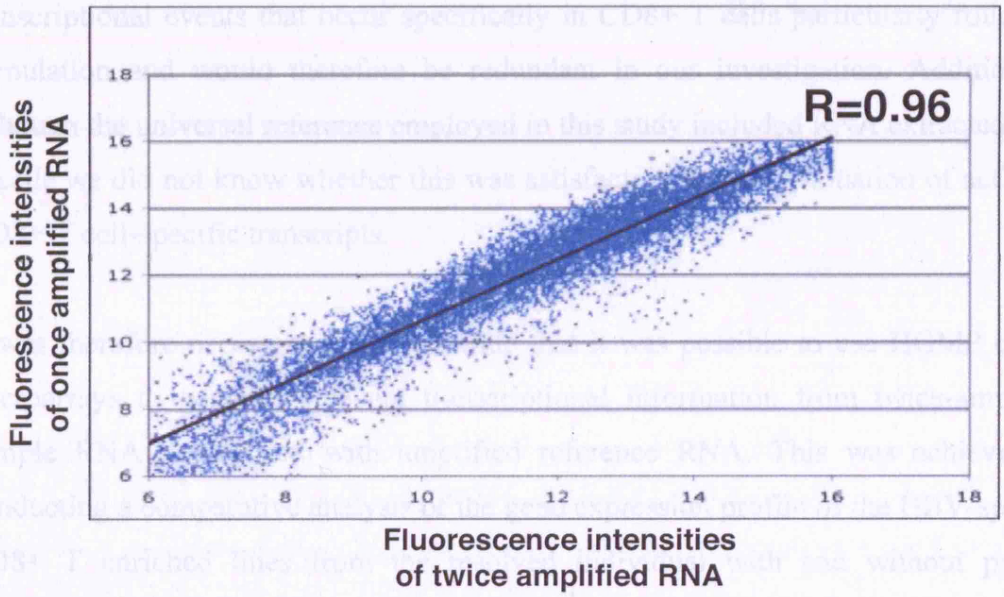

Figure 4.7
Comparison of once and twice amplified aRNA.
The influence of an additional round of amplification was evaluated by plotting the fluorescence intensities of once and twice amplified reference RNA that were labelled with Cy5 and Cy3 respectively and cohybridised to an HGMP cDNA array.

4.2.6 Primary evaluation of HGMP cDNA microarrays for HBV-specific CD8+ T cell analysis

4.2.6.1 Analysis of HBV-specific CD8+ T cell-enriched lines

HGMP cDNA arrays contained probes for genes expressed by multiple different cell types. Due to the inherent protein variability of different specialized cell types, it was likely that multiple genes on the array would be irrelevant to the analysis of transcriptional events that occur specifically in CD8+ T cells particularly following stimulation and would therefore be redundant in our investigation. Additionally, although the universal reference employed in this study included RNA extracted from T cells we did not know whether this was satisfactory for the evaluation of activated CD8+ T cell-specific transcripts.

It was therefore necessary to demonstrate that it was possible to use HGMP cDNA microarrays to generate relevant transcriptional information from twice-amplified sample RNA normalized with amplified reference RNA. This was achieved by conducting a comparative analysis of the gene expression profile of the HBV-specific CD8+ T enriched lines from the resolved individual with and without peptide restimulation at day 10.

5μg of twice amplified RNA from the resolved samples B-US and B-S were labelled with Cy5 and each were combined with 5μg of Cy3 labelled aREF and processed onto arrays. The data was extracted, normalized and log transformed as described previously. Although the amounts of input were standardized to 10μg/array (5μg of sample + 5μg of reference), subtle differences in hybridization, washing or labelling efficiency are expected. Normalization, with CLUSTER, compensated for these effects by transposing the data so that the medians overlapped (see section 1.13.2.6). This form of global normalization, also referred to as median centring, is based on the assumption that the majority of genes (>95%) do not significantly change their level

of expression and therefore the array data can be centred while still maintaining the relationships and the standard deviations within the data (Yang et al., 2002). Log_2 transformation forced the data into a normal distribution and enabled an interpretation where a Log_2 (Cy5/Cy3) ratio of 1 signified that the gene was up-regulated by a factor of 2 relative to the reference. In contrast, a gene with a Log_2(Cy5/Cy3) ratio of –1 was down-regulated by a factor of 2. Unchanged genes had a Log_2 (Cy5/Cy3) of 0 (Yang et al., 2002).

The comparative analysis of the normalized data indicated that several of the most highly expressed genes were those known to be highly relevant to immune function (listed in Table 4.1).

IFN-γ and TNF-α transcripts were found to be highly expressed in the short-term lines and both were up-regulated following antigenic restimulation in support of data generated at the protein level demonstrating that the intrahepatic inactivation of HBV by CD8+ T cells is achieved by these cytokines (Guidotti et al., 1996).

Granule exocytosis is a key effector mechanism used by CD8+ T cells to eliminate virally infected cells - granule serine proteases (granzymes) released at the immunological synapse enter target cells through perforin/granulysin-mediated pores and subsequently kill target cells principally by cleaving target substrates in the cytoplasm and in the nucleus (Ashton-Rickardt, 2005; Lieberman, 2003).
The cytokine lymphotoxin alpha is a member of the tumour necrosis factor family that is known to mediate a variety of inflammatory, immunostimulatory and antiviral responses (Banks et al., 2006; Ware, 2005).

IL-18 signalling activates virus-specific CD8+ T cells and drives them to produce IFN-γ (Raue et al., 2004). Signalling through the IL-18 receptor also promotes survival of activated cells through an increase in anti-apoptotic Bcl-2 that is also associated with an increase in the expression of IL-2 receptor beta chain (CD122) (Li et al., 2007).

The fact that several of the most highly expressed (and further upregulated genes) had well described roles in antiviral CD8+ T cell responses confirmed the validity of using HGMP arrays for the study of antigen-specific CD8+ T cells. It is important to note that despite the fact that the enriched samples that were analysed also contained transcripts from immune cells other than those that were HBV-core-specific CD8+ T cells, we were still able to detect key transcriptional events commonly associated with a virus-specific CD8+ T cell response.

Table 4.1: Selection of highly expressed genes with known immune function

Gene	**B-US** $[Log_2(Cy5/Cy3)]$	**B-S** $[Log_2(Cy5/Cy3)]$
Interferon-γ	4.8	7.6
TNF-α	4.9	7
Granzyme K	4.8	6.5
Lymphotoxin-a	1.8	4.3
Interleukin 18 receptor (α chain)	2.6	5.4
Interleukin 2 receptor (β chain)	4.1	6.3

4.2.6.2 Analysis of purified HBV-specific CD8+ T cells

Stimulated CD8+ T cells respond by exerting effector functions such as the secretion of cytokines like IFN-γ/TNF-α or modifying expression of cell surface signalling molecules. These soluble or cell-to-cell contact-dependent mechanisms have the potential of inducing non-specific bystander activation that could influence the data relevant to virus-specific CD8+ T cell responses. In order to control for this it we sought to complement the cumulative array data that was generated from whole short-term enriched lines with data from highly purified virus-specific CD8+ T cells. Two purification methods were adapted for the selection for HBV-specific CD8+ T cells from individuals with resolved and chronic infection.

4.2.6.3 Purification of HBV-specific CD8+ T cells associated with successful viral control

Multimer-labelling of HBV-specific CD8+ T cells from resolved individuals coupled to fluorescence activated cell sorting allowed for highly specific purification of virus-specific CD8+ T cells. This was an ideal method of purification as it is based on the selection of CD8+ T cells that express a TCR that is able to recognise a specific viral antigen.

10 million cells [with a core-specific CD8+ T cell enrichment of ~70% (of total CD8+ T cells)] were stained with an HLA-A2 HBV core 18-27 tetramer and cultured for a further 5 hours with the core 18-27 peptide. To ensure optimal staining tetramer-labelling was performed prior to the peptide stimulation because TCR expression can be down-regulated following activation (Kao et al., 2005). The engagement of TCRs by tetramers could have reduced the subsequent peptide stimulation by effectively blocking these from interacting with MHCI-presented peptides, however, tetramers themselves have the potential to activate complementary cells which was constructive to our experimental design.

From a total of 4.5 million acquired cells, 1.1 million were positively sorted at a purity of greater than 95%. Two rounds of *in vitro* transcription on mRNA extracted from these cells generated 16.2μg aRNA and was sufficient for the generation of a high quality array (figure 4.8 left).

In addition to flow cytometry based sorting, we were also able to obtain highly purified envelope- and core-specific CD8+ T cells from enriched lines (6 and 34 % of total CD8+ respectively) by magnetic column–based separation of pentamer labelled cells. Purities were 81% and 96% respectively (Figure 4.9).

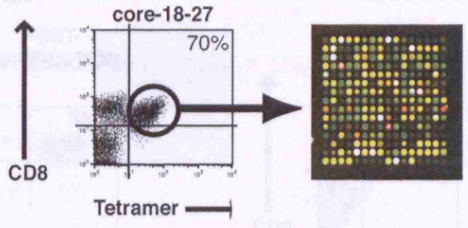

Figure 4.8
cDNA Microarray analysis of HBV-specific CD8+ T cells from a resolved individual
Core-specific CD8+ T cells were purified by flow cytometric sorting of a core-pentamer labelled short-term PBMC line from a resolved patient (left plot). mRNA extracted from the purified cells was then profiled by dual colour cDNA microarray technology (right).

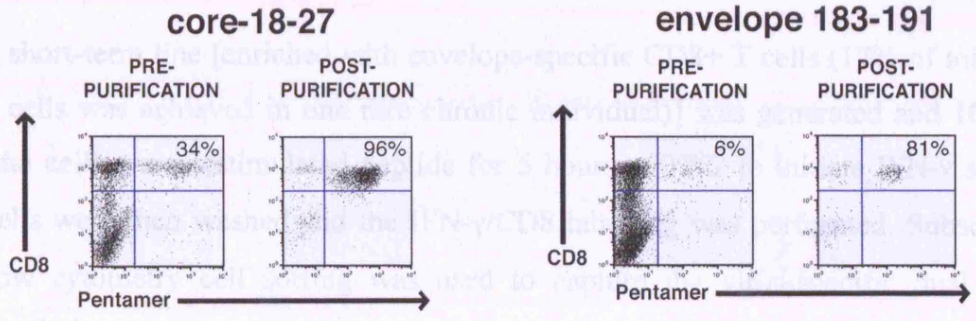

Figure 4.9
Magnetic purification of HBV-specific CD8+ T cells from a resolved individual. Short-term core 18-27 and envelope 183-191-enriched lines were stained with appropriate phycoerythrin(PE)-conjugated pentamers. Anti-PE magnetic beads were then used to positively select HBV-specific CD8+ T cells. Pre and post-purified samples were co-stained with anti-CD8 and analysed by flow cytometry.

4.2.6.4 Purification of HBV-specific CD8+ T cells associated with immune failure

Tetramers could not be used to purify HBV envelope-specific CD8+ T cells from individuals with chronic infection with high viral loads because these cells were not able to bind to the multimer (Reignat et al., 2002). However, these populations maintained their ability to produce IFN-γ in response to specific viral peptide stimulation allowing us to positively select these cells on the basis of effector function; see section 2.10 for further details.

A short-term line [enriched with envelope-specific CD8+ T cells (17% of total CD8+ T cells was achieved in one rare chronic individual)] was generated and 10 million total cells were restimulated peptide for 5 hours at 37°C to initiate IFN-γ secretion. Cells were then washed and the IFN-γ/CD8 labelling was performed. Subsequently, flow cytometry cell sorting was used to capture the virus-specific dual positive population.

From a total of 6.7 million acquired cells, $0.32x10^6$ envelope 183-91-specific CD8+ T cells were obtained. These were immediately lysed in an RNAse neutralizing mRNA extraction buffer and stored at –70°C. The two rounds of *in vitro* amplification generated 13.8μg of aRNA that was sufficient to produce a high quality array (figure 4.10).

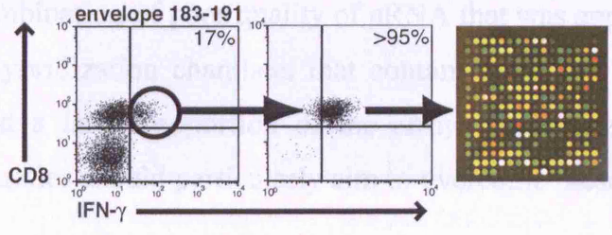

Figure 4.10
cDNA Microarray analysis of HBV-specific CD8+ T cells from an individual with chronic HBV infection.
Envelope-specific CD8+ T cells were purified by flow cytometric sorting of a short-term PBMC line from a chronic patient (left and middle plot); HBV-specific CD8+ T cells were labelled for IFN-γ with the IFN-γ secretion assay following a five hour peptide stimulation. mRNA extracted from the purified cells was then profiled by dual colour cDNA microarrays technology (right).

4.2.7 Selection of arrays generated in this study

Gene expression profiles were generated for a total of 48 arrays corresponding to 10 day PBMC lines (enriched for envelope 183-191- or core 18-27-specific CD8+ T cells) that were not peptide re-stimulated at day 10, peptide re-stimulated (for 6 hours) at day 10, and a limited number of samples that were re-stimulated at day 10 for either 1 or 16 hours.

We were unable to conduct an analysis of unstimulated versus stimulated samples due to low signal intensities or sub-optimal array images of the un-restimulated samples in particular because the data from substandard arrays were unreliable. This was due to a combination of poor quality of aRNA that was applied onto the arrays as well as leaky hybridization chambers that contaminated the hybridization mixture. Considering that a large proportion of the arrays were disqualified due to these factors, future studies should particularly aim to overcome these problems.

The 1 and 16 hour restimulated arrays were also not of appropriate quality; the lack of a continuous set of unstimulated and 1, 6, and 16 hour-stimulated samples from one individual did not permit a time course analysis to evaluate the dynamics of gene transcription changes. As mentioned previously, immediate early as well as late transcriptional events could provide important information regarding the effectiveness of the antiviral response and deserves, along with the unstimulated versus stimulated samples, to be studied in the future.

We were however able to generate high quality data from 19 arrays corresponding to peptide re-stimulated 10 day PBMC lines that were enriched for HBV-specific CD8+ T cells. The array data generated from these consisted of twelve resolved samples, six samples with high-level chronic HBV infection and one sample from an individual with low-level chronic infection. Specifically, they included seven core 18-27 enriched lines, eight envelope 183-91 enriched lines, purified core 18-27-specific

CD8+ T cells from two patients, and purified envelope 183-91-specific CD8+ T cells from an additional two patients (see Table 4.2 below).

Table 4.2: Details of the chronic and resolved samples processed onto cDNA microarrays

All samples were restimulated for 5 hours with cognate peptide prior to lysis

ARRAY	PATIENT	VIRAL DETERMINANT	SAMPLE TYPE	% IFN-γ+ (of CD8+ T)
1	R1	Core 18-27	enriched line	34
2	R1	Core 18-27	purified	96
3	R3	Core 18-27	enriched line	3
4	R11	Core 18-27	enriched line	10
5	R6	Core 18-27	enriched line	12
6	R10a	Core 18-27	enriched line	20
7	R10b	Core 18-27	purified	95
8	R10b	Core 18-27	enriched line	70
9	R1	Envelope 183-191	enriched line	6
10	R1	Envelope 183-191	purified	81
11	R6	Envelope 183-191	enriched line	10
12	R10a	Envelope 183-191	enriched line	5
13	C15	Core 18-27	enriched line	9
14	C16a	Envelope 183-191	enriched line	4
15	C16b	Envelope 183-191	enriched line	4
16	C22	Envelope 183-191	enriched line	1
17	C9a	Envelope 183-191	enriched line	3
18	C9a	Envelope 183-191	purified	95
19	C9b	Envelope 183-191	enriched line	3

4.2.8 Pre-analysis data processing

4.2.8.1 Selection of essential data

In addition to the large panel of relevant intracellular genes, each HGMP array dataset contained a significant quantity of data that was irrelevant to the CD8+ T comparative analysis. These redundant genes included several housekeeping genes, blank cells, positive and negative controls (Table 4.3/ figure 4.11/ for a representative example of positive and negative genes).

Although the mean Cy5 and Cy3 fluorescence intensities of the negative controls were used to filter-out data that fell below background levels, the redundant genes were subsequently not required for the comparative analysis between resolved and chronic samples and were therefore manually deleted from the master dataset. The individual reduced array datasets were then assimilated into a single excel file termed Dataset1-Reduced (D1-R) containing fluorescence intensities of approximately 5088 genes, in duplicate.

Table 4.3: Details of the negative and positive (bold) control genes spotted onto the HGMP cDNA arrays

Gene number	Gene number	Category
1	Yeast Intergenic Region from Chrom XI	dynamic range control 1
2	Yeast Intergenic Region from Chrom XI	dynamic range control 2
3	Yeast Intergenic Region from Chrom VII	dynamic range control 3
4	Yeast Intergenic Region from Chrom VII	dynamic range control 4
5	Yeast Intergenic Region from Chrom XII	dynamic range control 5
6	Yeast Intergenic Region from Chrom XII	dynamic range control 6
7	**Actin gamma-1**	**house keeping gene 1**
8	**Enoyl Coenzyme A hydrolase, short chain**	**house keeping gene 10**
9	**Glyceraldehyde-3-phosphate dehydrogenase**	**house keeping gene 2**
10	**Ubiquinol-cytochrome c reductase core pr**	**house keeping gene 3**
11	**Casein kinase II beta polypeptide**	**house keeping gene 4**
12	**EST highly similar to NY-REN-37 antigen**	**house keeping gene 5**
13	**Human hydroxymethyl glutaryl-CoA lyase**	**house keeping gene 6**
14	**Neuroblastoma RAS viral oncogene homolog**	**house keeping gene 7**
15	**Eukaryotic translation initiation factor**	**house keeping gene 8**
16	**Ubiquinol-cytochrome c reductase core pr**	**house keeping gene 9**
17	Arabidopsis thaliana protein G1p	negative control 1
18	Poly-dA oligonucleotide	negative control 2
19	Spotting buffer	negative control 3
20	Bacillus subtilis gene	negative control 4
21	Bacillus subtilis gene	negative control 5
22	**Total human genomic DNA**	**positive control**
23	Yeast Intergenic Region from Chrom XII	ratio control 1
24	Yeast Intergenic Region from Chrom XII	ratio control 2
25	Yeast Intergenic Region from Chrom XII	ratio control 3
26	Yeast Intergenic Region from Chrom XIII	ratio control 4

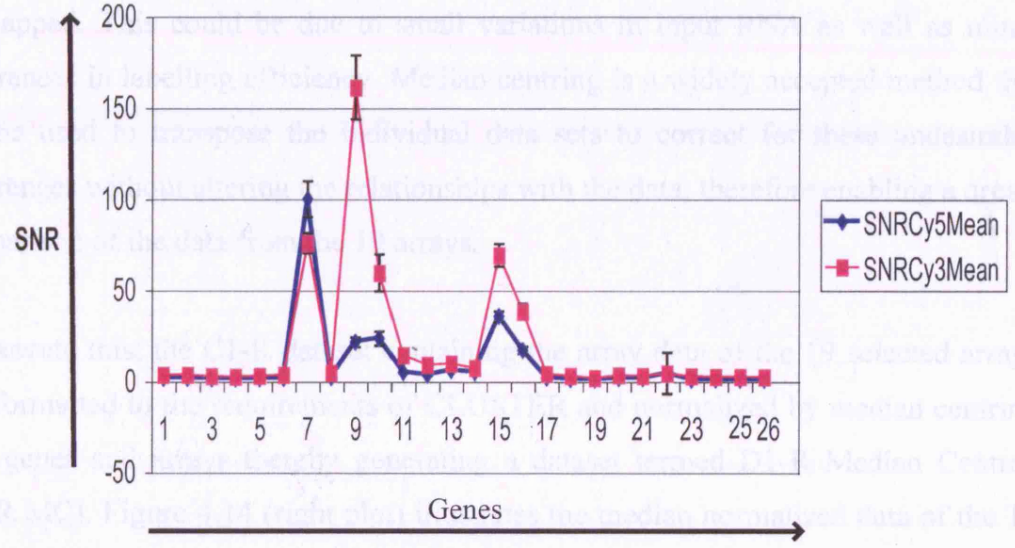

Figure 4.11
cDNA microarray negative and positive control genes.
cDNA microarrays contained 26 negative and positive control gene spots; the mean value of the former group was used to assess the non-specific background fluorescence of the array.

4.2.8.2 Data normalization

Every gene within an array that passed the filtration criteria was associated with an expression value (Log_2 Cy5/Cy3). Plotting the frequency of gene expression measurements per array produced a bell shaped curve indicating that the data is normally distributed (figure 4.15 left plot). Most of the data lay within the 95th percentile centred in close proximity to zero indicating that the majority of the genes did not change expression. However, the individual array datasets exhibited slight differences in median expression as indicated in the shifts of the plots when overlapped. This could be due to small variations in input RNA as well as minor differences in labelling efficiency. Median centring is a widely accepted method that can be used to transpose the individual data sets to correct for these undesirable differences without altering the relationships with the data, therefore enabling a cross-comparison of the data from the 19 arrays.

To execute this, the C1-R dataset containing the array data of the 19 selected arrays was formatted to the requirements of CLUSTER and normalized by median centring both genes and arrays thereby generating a dataset termed D1-R Median Centred (D1-R MC). Figure 4.14 (right plot) illustrates the median normalized data of the 19 arrays.

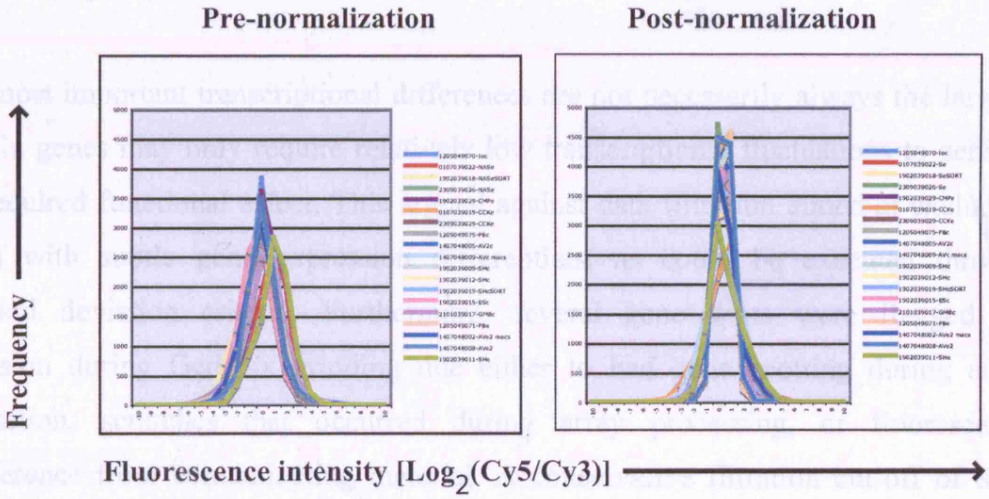

Figure 4.12
Normalization of array data - median centring.
In order to enable a comparative analysis of the gene expression data from the 19 individual arrays, the datasets were compiled into a single file and were collectively median centre-normalized with CLUSTER. Plots illustrate the distribution of the data of the individual arrays pre- and post-normalization.

4.2.8.3 Data filtration

Data filtration is a component of post-normalization pre-analysis data processing that is used to increase the stringency of the dataset. CLUSTER was capable of two forms of filtration: first, the exclusion of genes with inter-array expression values falling below a user-defined standard deviation threshold (thereby eliminating genes with small variations in expression across arrays) and second, the exclusion of genes for which array data was not available, the latter being adjusted according to percent of data entries present.

The most important transcriptional differences are not necessarily always the largest. Certain genes may only require relatively low transcriptional fluctuations to achieve the required functional effect. This argued against data filtration aimed at excluding genes with subtle gene expression differentials, as could be executed through standard deviation criteria. Furthermore, several gene spots were flagged for exclusion during Genepix gridding due either to bad gene spotting during array production, scratches that occurred during array processing, or fluorescence interference from contaminating material. A conservative filtration cut-off of 80% resulted in the loss of 1067 genes, 10% of the total dataset, introducing a substantial risk of over-looking critical genes. Thus, in order to minimize the incidence of false-negatives, an unfiltered dataset (D1-R MC UF) consisting of all 10166 data entries was utilized in the comparative analysis.

4.2.9 Data analysis

4.2.9.1 SOMs, hierarchical clustering and data visualization

In the first phase of the data analysis we decided to explore the relationship between the 19 individual samples according to the global gene expression profile without forcing a compartmentalization of the samples according to disease outcome (resolved versus chronic). To achieve this, CLUSTER (Eisen et al., 1998) was first used to generate self-organized maps and perform average linkage clustering of genes and arrays thereby arranging the individual arrays and genes according to similarity of the global gene expression profile. TREEVIEW (http://rana.lbl.gov/EisenSoftware.htm) was used to visualize the CLUSTER-organized data. This complementary PC-based bioinformatics tool generated a heat map in which colour intensity reflected the relative gene expression - red and green corresponding to up- and down-regulation respectively. An accompanying dendogram was produced that illustrated the similarity between the individual arrays as well as genes.

We observed that the primary dataset (D1-R MC UF) branched into two main groups (Figure 4.16; C and R). The majority of samples in group C (6/8) and group R (10/11) derived from individuals with chronic and resolved infection respectively. The single chronic patient whose HBV-specific CD8+ T cell gene expression branched with the resolved patients was the only chronic HBV patient included in this analysis that did not have high HBV DNA (greater than 10^6 IU/ml) and may therefore explain the aberrant segregation. It is unclear why two resolved samples segregated with the chronic samples in group C but could be due to a gene expression differential that was relatively quite low resulting in a large proportion of entries that appeared unchanged (black on the heat-map). Nonetheless, based on the overall sequestration, it was apparent that differences in gene expression between individual samples that

were induced following HBV antigen-specific activation with peptide were sufficient to differentiate samples according to the clinical outcome.

Among all the genes analysed (5088 genes per sample, spotted in duplicate), a clearly distinguishable cluster of genes was upregulated in chronic samples and down-regulated in resolved samples (compared to reference RNA, figure 4.15, blue box). Closer inspection identified a sub-cluster of genes that were functionally-related and exhibited a consistent divergence of expression level between the two groups. Of the 20 genes (with known functions) in this terminal sub-cluster, 16 had previously been described to participate in apoptosis (highlighted in red). 10 of these were found to overlap with highly significant genes short-listed by the SAM analysis (see section 4.2.9.2).

Within the 20 gene subcluster, transforming growth factor beta (TGF-β), an extremely potent inhibitor of lymphocyte function (Lotz et al., 1990) that has been shown to suppress several virus-specific CD8+ T cell associated with persistent infection including HCV, HIV and HTLV-1 (Alatrakchi et al., 2007; Garba et al., 2002; Kekow et al., 1990; Nagai et al., 1995), was also transcriptionally upregulated in the chronic samples, alluding to a similar involvement of this cytokine in chronic HBV infection. Additionally, Bid is a well-described mediator of cell death that functions by inducing membrane permeabilization following caspase cleavage (Milhas et al., 2005; Reiners et al., 2002; Stoka et al., 2001) displayed a similar gene expression differential.

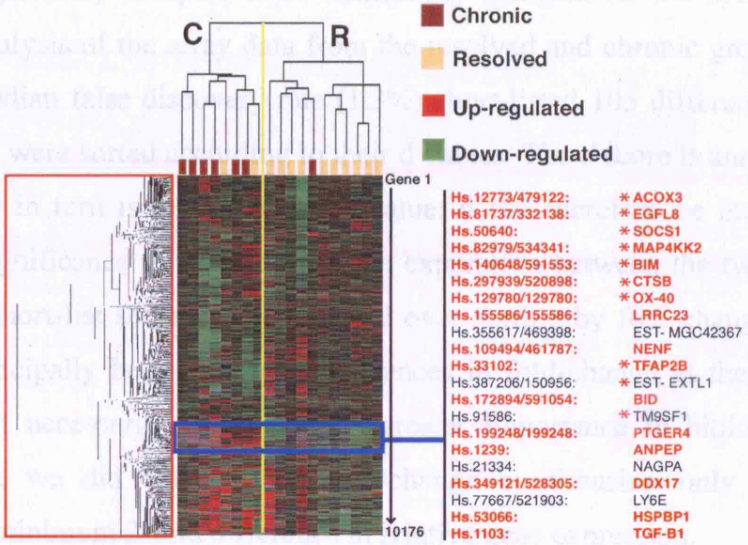

Figure 4.13
Visualization of cDNA microarray data processed by CLUSTER.
Treeview analysis of average linkage hierarchically clustered (with self-organized mapping) gene expression data. The top dendogram represents the similarity between individual arrayed samples (vertical plane) based on the global gene expression profile; a yellow line segregates the main clusters (C and R). The left dendogram (boxed in red) illustrates the clustering pattern according to similarity of expression between different genes. The blue box highlights a section of the heat-map where a group of genes exhibited marked upregulation in branch C compared to R (listed with original and current unigene references on the right; genes in red participate in apoptosis; genes overlapping with the SAM short-list are indicated by asterisks).

4.2.9.2 Significance analysis of microarrays (SAM)

SAM was used to process normalized (median centred) cDNA array data to identify genes with statistically robust differences in expression; see section 1.14.1. A selection of significant genes was determined by adjusting a user-defined tuning parameter (Δ) that is related to a false discovery rate (q) (Tusher et al., 2001); a FDR of <5% is generally accepted to be sufficiently stringent for the SAM analysis. An unpaired analysis of the array data from the resolved and chronic groups of samples at a low median false discovery rate (1.3%) short-listed 105 differentially regulated genes; these were sorted according to their d values. The d score is analogous to the t-statistic that in turn is related to the p value; it can therefore be interpreted as the statistical significance of differential gene expression between the two groups. This method of short-list sorting was preferred over sorting by fold-change (as a second option) prinicipally because larger differences in fold-change at the transcriptional level do not necessarily translate to a greater importance in biological function. Nonetheless, we did consider the fold-change by focusing only on genes that exhibited a minimum 2-fold difference in relative gene expression.

76 genes had increased and 29 genes had reduced expression, in chronic compared to resolved HBV-specific CD8+ T cell samples (figure 4.14, table 4.4 & table 4.5). We observed that the up-regulated genes in HBV-specific CD8+ T cells from patients with CHB exhibited larger fold-change increases, ranging from 2 to 9 fold. In corroboration with our findings from the hierarchical cluster analysis, a large number of the genes that were short-listed by SAM were also found to participate in apoptotic events. More importantly, multiple apoptosis-related genes from the two independently generated short-lists overlapped (figure 4.13, asterisked), confirming that a group of functionally-related genes were transcriptionally altered in antigen-specific CD8+ T cells in chronic HBV infection.

Figure 4.15 illustrates a sample of five genes that were selected by both methods of analysis, with mean and standard deviation of expression levels relative to the

reference RNA (Mann Whitney test). Bim has been shown to drive the deletion of an immunodominant CD8+ T response in a persistent viral infection (Grayson et al., 2006). The forkhead transcription factor (G1B), shortlisted in the SAM analysis, was also elevated in the responses associated with CHB; forkhead transcription factors have been associated to the regulation of Bim (Behzad et al., 2007; You et al., 2006). Forkhead transcription factors are in turn related to GADD45-γ (growth arrest and DNA-damage inducible response gene) that is upregulated in the antiviral response in CHB; GADD45-γ contributes to effector T lymphocyte death (Liu et al., 2005c; Zerbini and Libermann, 2005). Additionally, supporting the participation of Bim-associated apoptosis, gene expression levels of the tumor necrosis factor receptor (TNFFR)-associated factor 1 (TRAF-1) (shortlisted by SAM) were relatively lower in the responses associated with CHB; TRAF-1 inhibits antigen-induced apoptosis of CD8+ T lymphocytes (Sabbagh et al., 2006; Speiser et al., 1997). The participation of these four inter-dependent functionally-related genes supported the involvement of an apoptotic mechanism.

With respect to the other four sample genes depicted in Figure 4.15, Cathepsin B has been shown to mediate the apoptosis of T lymphocytes (Michallet et al., 2003), particularly those that have been subject to supraoptimal activation (Michallet et al., 2004) suggesting involvement in high dose tolerance. Ox-40 has been shown to promote Bcl-Xl and Bcl-2 expression (Rogers et al., 2001). SOCS1 is a negative regulator of cytokine signalling and impairs proliferation of T cells (Trop et al., 2001). MAP4KK2 activates MAP kinases resulting in a selective deletion of CD8+ T cells (Merritt et al., 2000). For each of these genes, the transcripts were consistently raised in HBV-specific CD8+ T cells from CHB, both in highly purified populations and enriched 10 day lines. These genes showing highly significant differences (increases) in HBV-specific responses from chronic compared to resolved patients are all involved in apoptotic pathways that can lead to lymphocyte death. Thus, we have shown that data from two independent analyses highlighted overlapping clusters of apoptosis-related transcripts that were dysregulated in the HBV-specific CD8+ T cell response associated with chronicity.

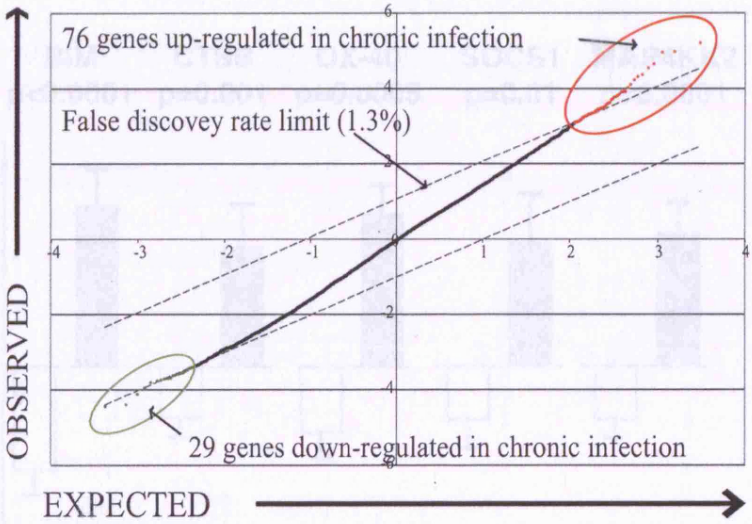

Figure 4.14
Statistical analysis of cDNA microarray data.
Significance Analysis of Microarrays (SAM) plot illustrating the most significant differentially regulated genes (false discovery rate - 1.3%) between the group with chronic and resolved HBV infection.

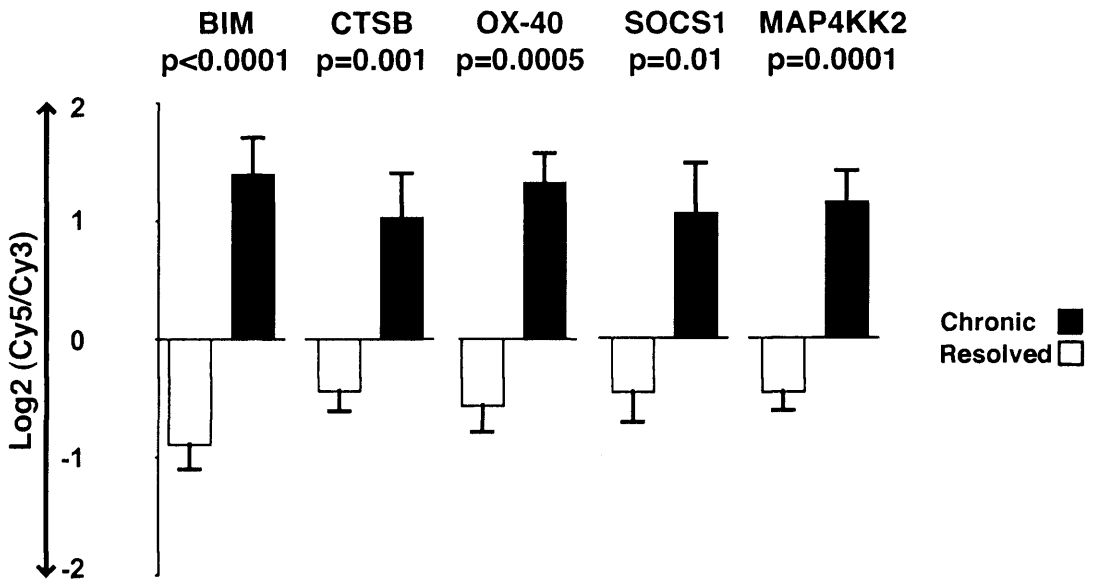

Figure 4.15
Selection of differentially regulated genes.
cDNA array data of the mean transcriptional levels (including S.E.M. limits) of five apoptosis-related genes in HBV-specific CD8+ T cell responses in resolved and chronic infection with stastical significance (Mann Whitney test) as indicated.

Table 4.4

SAM analysis - 76 genes up-regulated in chronic infection.

76 positive significant genes

Gene Name	Unigene ID (Hs.) *	Score(d)	Fold Change
Bcl-2 interacting mediator	*140648*	*5.20*	*6.63*
EST	330442	4.97	4.81
Complement component 3	1203320	4.96	5.81
Complement component 3	1203320	4.64	5.08
Secreted frizzled-related protein 1	7306	4.54	3.75
N-acetylglucosaminidase, alpha	50727	4.37	4.09
Myelin associated glycoprotein	1780	4.32	8.56
Insulin-like growth factor binding protein	77326	4.23	9.25
Extracellular matrix protein 2	35094	4.09	3.08
Coiled-coil domain containing 52	477144	4.02	4.42
Growth arrest & DNA-damage-inducible, gamma	9701	3.91	4.15
Contactin	143434	3.91	2.79
Frizzled homolog 2	81217	3.90	2.86
Serine (or cysteine) proteinase inhibitor, clade A	297681	3.84	3.74
Cytochrome c oxidade subunit Va	434076	3.80	3.68
Ki-ras	ND	3.77	3.57
Dystrobrevin, alpha	336678	3.76	2.23
Integrin, alpha 6	227730	3.72	4.22
Procollagen (type III) N-endopeptidase	183138	3.70	2.69
Ribosomal protein S4, Y-linked	180911	3.62	3.48
Matrix metalloproteinase 17	159581	3.62	4.28
Forkhead box G1B	386249	3.60	3.32
Matrix metalloproteinase 13	2936	3.57	2.98
Connective tissue growth factor	75511	3.52	7.34
Cathepsin B	*297939*	*3.50*	*3.33*
Profilin 1	75721	3.49	4.12
Chloride channel 4	199250	3.47	2.77
Suppressor of cytokine signalling	*405946*	*3.47*	*4.51*
Uracil-DNA glycosylase	78853	3.45	2.49
Pyrroline-5-carboxylate reductase 1	79217	3.45	3.50
Gamma-aminobutyric acid (GABA) A receptor	45740	3.44	3.40
STAT6	ND	3.42	2.84
Cut-like 1, CCAAT displacement protein	147049	3.41	2.74
OX-40	*129780*	*3.40*	*3.00*
Rho GTPase-activating protein	111138	3.37	4.11
Lysophosphaphatidic acid G-protein-coupled R.2	75794	3.37	3.31
Tryptase beta 1	405479	3.36	3.09
Apolipoprotein A-I	93194	3.35	7.50
Mitogen-activating protein kinasex4 2	*82979*	*3.34*	*3.34*
Catenin (cadherin-associated protein)	80220	3.34	3.30
Acyl-Coenzyme A oxidase 3, pristanoyl	12773	3.33	3.39
Folate receptor 2	24194	3.32	2.94
DNA (cytosine-5-)-methyltransferase 1	202672	3.31	4.62
Glycine receptor, beta	32973	3.31	4.68

EST	(N55083)	3.31	3.13
Gap junction protein, beta 1	333303	3.29	3.80
EST	(R59352)	3.28	2.67
EST	(R37350)	3.27	3.11
Amyloid B (A4) precursor protein-binding m.3	17528	3.26	3.87
Human insulin-like growth factor binding	635441	3.26	6.13
Cysteine-rich, angiogenic inducer, 61	8867	3.25	4.89
Transcription factor 21	78061	3.25	3.13
Hypothetical protein LOC27351	570455	3.23	4.16
Chromosome X open reading frame 12	23119	3.22	3.03
Ribosomal protein S4, Y linked	180911	3.22	3.08
Ras homolog gene family, member E	6838	3.21	3.52
Frizzled homolog 4	19545	3.19	3.80
Hypothetical protein LOC138046 isoform 1	121663	3.18	2.33
Transcription factor AP-2 beta	33102	3.17	3.33
MET	ND	3.16	3.31
Fibroblast growth factor 2	284244	3.15	4.01
Suppressor of cytokine signaling 1	50640	3.15	3.45
EST	(AI356286)	3.13	3.04
Baculoviral IAP repeat-containing 3	127799	3.13	4.51
Palmitoyl-protein thioesterase 2	81737	3.12	3.75
Lactotransferrin	105938	3.12	2.92
Angiopoetin 1	ND	3.12	3.14
Acyl-Coenzyme A oxidase 3, pristanoyl	12773	3.11	3.32
Telomerase-associated protein 1	232070	3.11	3.47
Rho/Rac guanine nucleotide exchange factor 2	337774	3.11	3.27
Secreted frizzled-related protein 1	7306	3.11	2.49
D component of complement (adipsin)	155597	3.11	2.27
OX-40	129780	3.09	3.00
Solute carrier family 3	239106	3.09	4.24
EST	(R53362)	3.09	2.30
EST	(R32170)	3.09	3.15

ND = not defined; EST = expressed sequence tag; * (Genebank ID provided where Unigene ID unavailable)

Table 4.5

SAM analysis - 29 genes down-regulated in chronic infection.

29 negative significant genes

Gene Name	Unigene ID (Hs.) *	Score(d)	Fold Change
Diacylglycerol O-acyltransferase homolog 1	288627	-4.44	0.33
GTP-binding protein Rho7	603111	-4.39	0.29
Interferon, gamma	856	-4.12	0.16
Sjogren's syndrome nuclear autoantigen 1	18528	-3.95	0.43
Zinc finger RNA binding protein	173518	-3.80	0.32
E2F transcription factor 1	96055	-3.77	0.30
Huntingtin interacting protein	107019	-3.74	0.45
Human D9 splice variant B mRNA, complete	37616	-3.71	0.35
Flightless I homolog	83849	-3.70	0.40
Hypothetical protein MGC14480	37616	-3.69	0.37
Hypothetical protein FLJ10432	143187	-3.69	0.37
Fibroblast growth factor i.c. binding protein	7768	-3.63	0.46
Phosphomevalonate kinase	30954	-3.62	0.42
Growth arrest & DNA-damage-inducible, alpha	80409	-3.59	0.38
Integrin beta 3 binding protein	82084	-3.58	0.40
Flightless 1 homolog	83849	-3.57	0.37
ATP-binding cassette, sub-family F	153612	-3.55	0.52
Vestigial like 1	9030	-3.55	0.50
CD37 antigen	153053	-3.54	0.27
Glycine dehydrogenase	380791	-3.52	0.27
Phosphorylase kinase, gamma 2	196177	-3.49	0.39
SET domain containing 1A	297483	-3.46	0.30
Hypothetical protein XP_375359	97805	-3.43	0.36
Hypothetical protein MGC5356	197755	-3.43	0.35
Postreplication repair protein hRAD18p	21320	-3.42	0.33
KIAA0368 protein	3852	-3.40	0.45
Phosphorylase, glycogen	75658	-3.40	0.38
Integrin-linked kinase	6196	-3.40	0.38
Small nuclear ribonucleoprotein polypeptide A	173255	-3.39	0.46

ND = not defined; EST = expressed sequence tag; * (Genebank ID provided where Unigene ID unavailable)

4.3 Discussion

The main obstacle in our understanding of the failure of HBV-specific CD8+ T cells in chronic viral infection has been the inability to obtain a sufficient number of these antiviral populations to conduct a broader analysis. Previous studies have focused on the analysis of individual phenotypic or functional traits such as migration or activation markers, cytokine receptors, cytokine secreting capacity, perforin content, cytolytic capacity or proliferative potential (Bengsch et al., 2007; Bertoletti et al., 1997a; Kaech et al., 2003; Lord et al., 2000; Maile et al., 2005; Paiardini et al., 2005; Penna et al., 2007; Xiong et al., 2001), often extending observations reported in other persistent viral infections. However, a comprehensive and holistic study of the mechanisms underlying HBV-specific CD8+ T cell dysfunction has not been possible. A better understanding of these processes would open up new possibilities for therapeutic intervention aimed at reconditioning this failing arm of the immune response.

Although gene expression profiling had been used to study immune cells (Ji et al., 2003; Palena et al., 2003; Raghavan et al., 2002; Zhang et al., 2002), it had not been applied for the investigation of human virus-specific CD8+ T cells, with the exception of a recent study into HTLV-1 infection (Vine et al., 2004). Vine, A.M. et al successfully managed to apply Affymetrix genechip technology to analyse HTLV-1 specific responses in two group of patients; those with low or high proviral load. They demonstrated that circulating CD8+ T cells in the group with low-proviral load over-expressed several genes involved in cell-mediated lysis or antigen recognition, and proposed that viral suppression was associated with this stronger CD8+ T cell cytolytic activity. This work was of particular importance to our study because CD8+ T cells were profiled from patients directly *ex vivo*; these samples contained low frequencies of HTLV-1-specific CD8+ T cells, yet they were able to detect transcriptional differences of key genes involved in CD8+ T cell antiviral function.

HBV-specific CD8+ T cells circulate at frequencies 10-100 fold less than the HTLV-1-specific CD8+ T (Bieganowska et al., 1999; Greten et al., 1998; Maini et al., 1999;

Nagai et al., 2001; Utz et al., 1996). We therefore decided to further enhance the probability of detecting critical differences in anti-viral effector function by selectively enriching HBV-specific CD8+ T cells by short-term culture and then specifically activating these antiviral populations prior to gene expression analysis. Although short-term culture was not ideal due to the possibility of altering the populations of concern, studies have indicated that the dysfunction is preserved when expansion is performed with only a single round of peptide stimulation; for example, the phenotypic defect of tetramer negativity was unchanged (Reignat et al., 2002). Additionally, we overcame the main limitation of sample size by applying an amplification procedure to linearly increase messenger RNA transcripts in activated HBV-specific CD8+ T cells, and subsequently confirmed that these amplification procedures did not deviate the fidelity of the gene expression data. By profiling as little as 300,000 PBMCs containing HBV-specific cells (at 1-70% of the total CD8+ T cell populations), we were able to detect the transcriptional up-regulation of several immunologically relevant genes, thereby confirming the validity of this novel technology (as well as the particular type of microarrays selected for this study) for the analysis of antigen-specific CD8+ T cells. We were subsequently able to process multiple samples of activated HBV-specific CD8+ T cells, utilising bulk enriched lines or following highly selective purification, from patients with resolved or chronic infection.

Thus, the major contribution to the field has been the application of a pre-existing technology for the study of limited numbers of human immune cells. Although flow cytometry has been previously used successfully to study relatively small numbers of virus-specific CD8+ T cells, only a few parameters could be assessed simultaneously; these were usually limited to the availability of corresponding monoclonal antibodies. With cDNA microarrays, we simultaneously screened thousands of intracellular genes.

Our strategy was to compare two groups of samples - effective and ineffective responses – and focus on genes that exhibited differences in expression that had strong statistical significance, thereby providing candidate genes for more through

studies. This had the potential of opening up the possibility of identifying entirely novel genes involved in viral persistence in the context of HBV infection. Furthermore, as other viral pathogens may survive through the evolution of anti-immune mechanisms similar to those employed by HBV, the findings of this investigation promised to open-up important new avenues to study novel aspects of failed immune responses associated with other persistent microbes such as HIV, HCV, EBV and CMV.

In this work, two independent methods of analysis were used to demonstrate that a group of genes were up-regulated in HBV-specific CD8+ T cell responses associated with chronic infection. Several of these have well defined key roles in cellular apoptosis suggesting that functionally–related mechanisms were contributing to sub-optimal effector CD8+ T cell activity and thereby permitting viral persistence.

We propose that activated HBV-specific CD8+ T cells associated with persistent infection are highly prone to apoptosis compared to their counterparts in resolved individuals and this is likely to contribute to the hyporesponsiveness that is observed in chronic infection as reported in the previous chapter. Further studies were required to validate these findings and subsequently individually investigate promising candidate genes to better understand their role (at the protein level) in HBV-specific CD8+ T cell dysfunction.

5 CHAPTER 5

An investigation of Bim-mediated HBV-specific CD8+ T cell attrition.

5.1 Background

In the preceding chapter, cDNA microarray technology was optimized to enable an analysis of the effector response of HBV-specific CD8+ T cells following their activation with viral peptides. We found a marked up-regulation of several genes in the antiviral CD8+ T cell response in persistent infection as compared to responses in individuals that spontaneously resolved the virus. A large proportion of the short-listed genes were known to be involved in cellular apoptosis and this suggested that the former populations were preferentially being driven towards death. These data were in line with the marked HBV-specific CD8+ T hypo-responsiveness associated with chronic HBV infection that was reported in chapter 3.

To continue these investigations, we focused our efforts on the Bcl2-interacting mediator Bim (O'Connor et al., 1998) that belongs to the Bcl-2 family of pro-apoptotic- proteins (Huang and Strasser, 2000).

Of all the genes short-listed by our bioinformatics analyses (cref), the transcriptional differences of Bim, between the groups with resolved and chronic infection, had the highest statistical significance. This proapoptotic protein has been shown to play a critical role in the thymic and peripheral deletion of T lymphocytes (Bouillet et al., 1999; Bouillet et al., 2002; Hildeman et al., 2002). More importantly, it is involved in CD8+ T cell apoptosis during chronic viral infection. Grayson et al demonstrated that in contrast to wild-type mice where there was a decline of virus-specific CD8+ T cells following LCMV infection, Bim-deficient mice maintained these populations demonstrating the participation of this protein in the down-regulation of the antiviral response during persistent infection (Grayson et al., 2006).

Cross presentation of soluble antigen has recently been implicated in the peripheral deletion of CD8+ T cells through a Bim-mediated mechanism (Davey et al., 2002) (Kurts et al., 1997). Given that chronic HBV infection is associated with the secretion of large quantities of viral antigens, this suggested that Bim could be involved in the attrition of the HBV-specific CD8+ T cell response in persistent infection.

There are three major isoforms of Bim, (EL, L and S) (O'Connor et al., 1998; O'Reilly et al., 2000); all of which contain a Bcl-2-homology region type 3 (BH3) (Boyd et al., 1995; Chittenden et al., 1995) that allows them to interact with the BH1 and BH2 regions of relevant anti-apoptotic proteins, particularily Bcl-2.
Six novel isoforms of Bim derived by alternative splicing were subsequently identified and designated Bima1, a2, b1-b4 (U et al., 2001) or alternatively, BimAC, ABC, AD, ACD, A and ABCD (Marani et al., 2002); none of these contain the C-terminal hydrophobic region and only Bima1 and Bima2 contain the BH3 domain (U et al., 2001).

This structural variety has made the mechanism of action difficult to understand. There are however two main processes that are thought to be involved: an indirect mechanism (via pro-survival proteins) and a direct mechanism; both converge on the multi-domain proapoptotic proteins, Bax and Bak. Dimerization of these effector molecules, following activation, allows them to form pores in the mitochondrial membrane resulting in the release of caspase-activating cytochrome c that eventually leads to nuclear destabilization and cell death.

Some studies have shown that Bim-mediated human T cell deletion occurs through gross increases in intracellular levels (Dijkers et al., 2000). Apart from death triggers initiated by specific stimuli though cell surface death-inducing receptors such as Fas/TNFRI (Goldstein et al., 2001; Hildeman et al., 2002; Nguyen et al., 2002b), TCR triggering can specifically upregulate of BimL&S (Sandalova et al., 2006; Sandalova et al., 2004). Expression above a relatively low threshold kills cells via the caspase pathway (Kumar, 2007; Sehra and Dent, 2006); inhibition of caspases antagonized Bim-induced cell death (O'Connor et al., 1998). Increases above this

threshold can however be tolerated when Bcl-2 expression is highly upregulated (O'Connor et al., 1998) supporting an indirect mode of action.

Another facet of Bim-mediated death involves translocation of the protein. Bim (EL/L and S) contain a C-terminal hydrophobic region that is important to its localization to intracytoplasmic membranes (Kroemer, 1997), independently of its association with Bcl-2. BimEL, the predominant isoform in T cells, is normally sequestered to microtubules via a direct interaction with tubulin but is released when phosphorylated – this liberated form interferes with anti-apoptotic Bcl-2. Furthermore, in the early phase of apoptosis, phosphorylted BimEL (pBimEL) is N-terminally cleaved by caspases resulting in a modified form that has higher affinity for Bcl-2; this can provide a positive feedback signal ensuring death of the cell (Chen and Zhou, 2004).

In contradiction to these findings, Marrack et al. argue that most of the intracellular Bim in activated T cells is localized to the mitochondria and not the microtubules (Zhu et al., 2004). They favor a mechanism of CD8+ T cell death that is caused by the drop in the levels of Bcl-2 that accompanies activation; this results in increases in free Bim that may participate directly in the activation of proapoptotic Bax and Bak (Harada et al., 2004). In support of this, the novel isoform BimAD, directly engages and activates proapoptotic Bax following heterodimerization with BimS (Marani et al., 2002), indicating that interactions with the Bcl-2 family of proteins can be bypassed. Additionally, a more recent study demonstrated that BimS expression was followed by its rapid translocation to the mitochondrial membrane where it initiated Bax activation independently of the Bcl-2 proteins (Weber et al., 2007).

The most recent studies have indicated that Bim does not have to engage Bax/Bak to initiate mitochondrial permeabilization. Instead, apoptosis is thought to be controlled because Bax/Bak is sequestered by pro-survival proteins and neutralization of the latter by Bim induces mitochondrial disruption (Uren et al., 2007; Willis et al., 2007). The major drawback of our cDNA microarray studies was that although the technology allowed an accurate quantification of the global pattern of mRNA

expression, it is known that gene transcripts are not necessarily translated into protein products. mRNA is subject to post-transcriptional regulation; intracellular microRNAs (miRNAs) can silence gene function either by repressing translation or promoting mRNA degradation (Behm-Ansmant et al., 2006; Liu et al., 2005a; Liu et al., 2005b; Rehwinkel et al., 2005).

It was therefore necessary to confirm that the transcriptional trends observed for Bim were present at the protein level. Furthermore, post-translation modifications are often required to extend the functionality of a protein either by the attachment of a large variety of functional groups (acetyls, phosphates, lipids, carbohydrates), by altering the chemical nature of individual amino acids, or by structural changes (formation of disulphide bonds or proteolytic clevage); these factors also have to be taken into consideration.

We were unable to conduct western blot analysis, that could address some of these issues, due to our inability to purify sufficient HBV-specific CD8+ T cells, therefore, we decided to further the study by focusing on quantitative differences of the Bim protein (isoform unrestricted) by flow cytometry. Given that a consensus regarding the mechanism of action of this protein has not as yet been reached, we also decided to examine the functionality of Bim in mediating death of HBV-specific CD8+ T cells by interfering with the death-pathway further downstream of the molecule at a stage that has been accepted. This work concludes with preliminary studies aimed at investigating the role of Bim in HBV-specific CD8+ T cell tolerance induced by antigen cross-presentation.

5.2 Results

5.2.1 Validation of microarray data by real-time quantitative PCR

The array data showed more Bim transcripts (6.6 fold upregulated) for samples from CHB patients than for the resolved samples. In order to assess whether the observed transcriptional difference of Bim expression was reliable and not an artifact of the cDNA microarray technology, we conducted a quantitative real-time PCR (QPCR) on RNA material reserved from four of the original samples used in the array analysis; two samples from resolved individuals and two samples from individuals with chronic infection (high viral load). This method is considered by experts to be the most appropriate method for the confirmation of microarray-generated data (Provenzano and Mocellin, 2007).

The comparative analysis of QPCR data involves normalization of each sample to a unique cellular gene that does not alter its level of transcription. Traditionally, housekeeping genes such as b-actin or GADPH have been employed, however, recent data indicate that these commonly used internal normalization genes do in fact exhibit subtle transcriptional fluctuations across different biological samples (Radonic et al., 2004; Thellin et al., 1999).

Data from our own cDNA microarray studies (normalized by global median centring) were in support of these findings, demonstrating that the transcriptional expression of several housekeeping genes exhibited significant variability between the 19 samples that were tested (figure 5.1).

Although these small changes are acceptable to gel-based assays such as northern blot analysis or RNAse protection assays they are not appropriate for QPCR normalization. This is mainly because the latter is a highly sensitive technology in which the smallest transcriptional differences have a large impact on the interpretation of data.

Recent studies have however shown that the human acidic ribosomal protein (HuPo) is an acceptable internal control gene; expression was the least variable of several reference genes tested (Dheda et al., 2004; Dheda et al., 2005). Furthermore, studies of a monocytic cell line exposed to varying stimuli (Ifn-g, IL-1 or TNF-a) indicated that HuPo was also least variable in comparison to the commonly used internal controls, namely b-actin, elongation initiation transcription factor 2A (EITF2A) and hypoxanthine phosphoribosyltransferase 1(HRPT) (unpublished observations, M. Fife, U.C.L.). For these reasons, HuPo was selected as the normalization factor for our QPCR analysis.

The Sybr green-based QPCR approach is commonly used in QPCR, however, a major weakness is that spurious non-specific PCR products contribute to a false-positive signal that leads to an overestimation of the gene-specific transcript of interest. This can on some occasions be minimized through optimizations of annealing temperature or magnesium concentrations in the PCR reaction mix. However, in order to rapidly execute this phase of the study with maximum specificity and accuracy (especially considering that only a limited number of the original samples were to be tested), an alternative method using commercially designed and pre-optimized gene-specific primers and an internal labelled probe were employed. We also applied relative quantitation as it overcame the need for a standard curve, the prerequisite for which would have been the generation, quantitation and optimization of a Bim-transcript control template.

Analysis of the quantity of Bim transcripts in the four samples tested demonstrated that the lowest quantity of Bim was detected in resolved sample 1 and this was 8 fold higher in resolved sample 2, but in comparison, the expression of Bim was found to be 28 and 29 fold higher in chronic sample 1 and 2 (Figure 5.2).

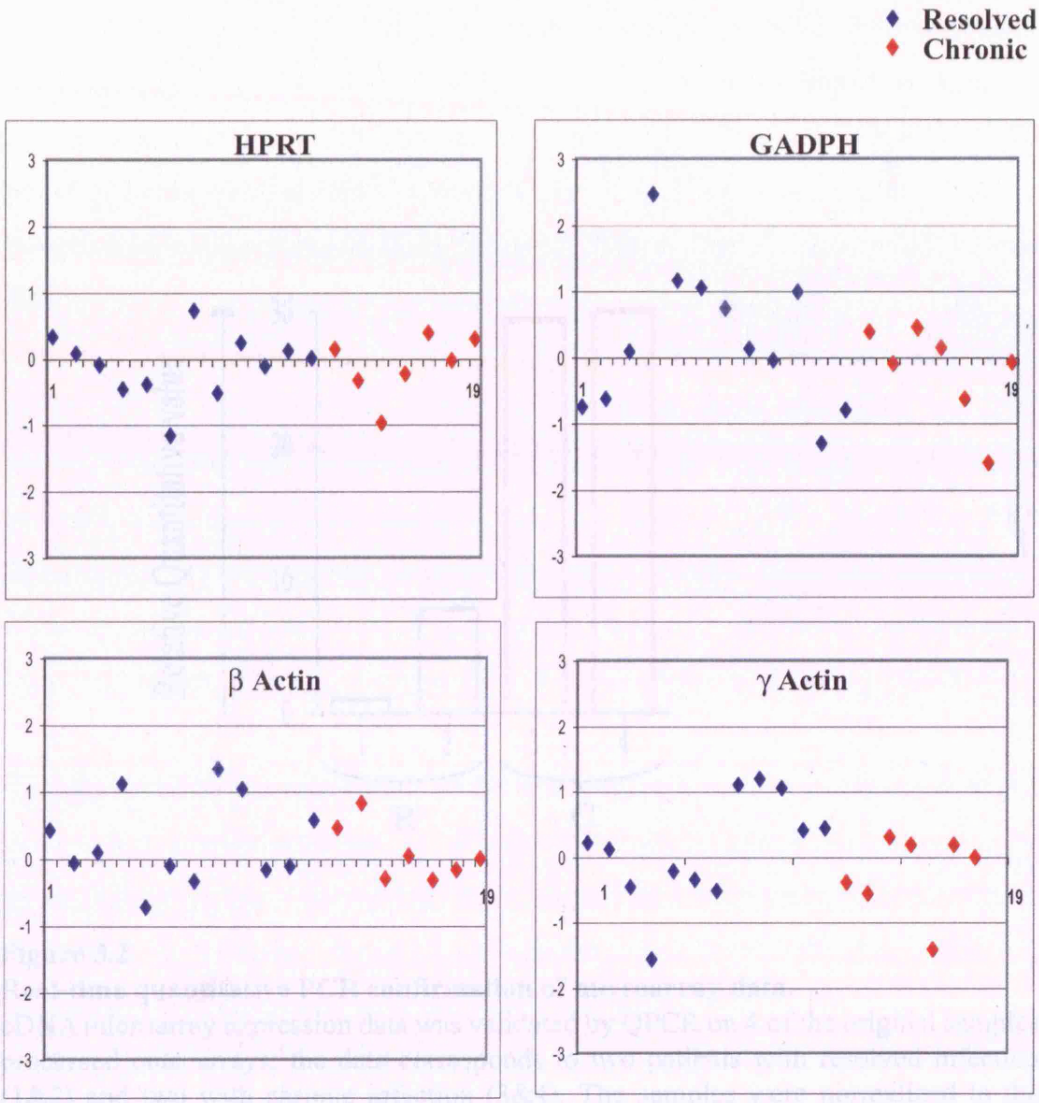

Figure 5.1
Transcriptional expression of 4 common housekeeping genes.
Normalized (median centred) cDNA array data of RNA transcripts for 4 internal housekeeping gens derived from 19 chronic and resolved samples.

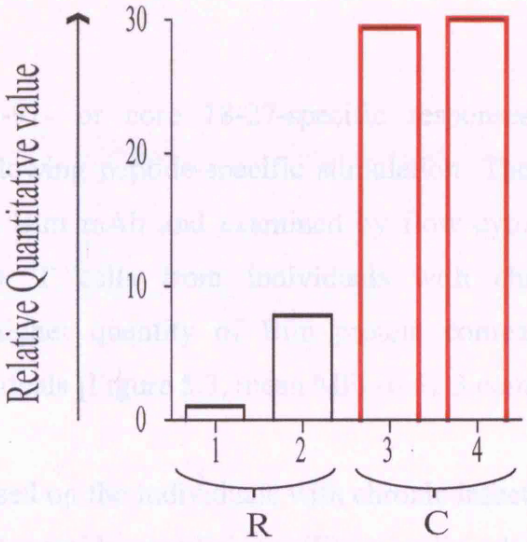

Figure 5.2
Real-time quantitative PCR confirmation of microarray data.
cDNA microarray expression data was validated by QPCR on 4 of the original samples processed onto arrays; the data corresponds to two patients with resolved infection (1&2) and two with chronic infection (3&4). The samples were normalized to the internal housekeeping gene (human acidic ribosomal protein) and expression was evaluated relative to the sample that contained the least Bim transcript (1).

5.2.2 Validation of differential Bim expression at the protein level

A cellular response to a stimulus is ultimately exerted through the activity of proteins. Although transcriptional increases are suggestive of subsequent increases at the protein level this is not necessarily always the case. It was therefore necessary to extend our studies and quantify Bim at the protein level. This was done in a larger sample of patients who had HBV-specific CD8+ T cell populations after 10 days *in vitro* expansion - 16 responses from resolved patients and 23 from CHB infected patients.

Envelope 183-91- or core 18-27-specific responses were identified by IFN-γ production following peptide-specific stimulation. These populations were also co-stained with a Bim mAb and examined by flow cytometry. We found that HBV-specific CD8+ T cells from individuals with chronic infection contained a significantly higher quantity of Bim protein compared to their counterparts in resolved individuals [Figure 5.3, mean MFI of 52.3 compared to 20.7; $p<0.0001$].

We then focussed on the individuals with chronic infection and a comparison of IFN-γ+ cells (viral peptide-specific) to IFN-γ- cells (those unable to recognise and therefore respond to the viral peptide) revealed that Bim expression was significantly higher in the former (mean MFI of 52.3 compared to 33.6; $p=0.008$) (figure 5.4).

Our data suggest that changes in the interactions of the Bcl-2 family due to an upregulation of Bim could be perturbing the delicate equilibrium that ensures cell survival and instead could be driving these populations towards apoptosis.

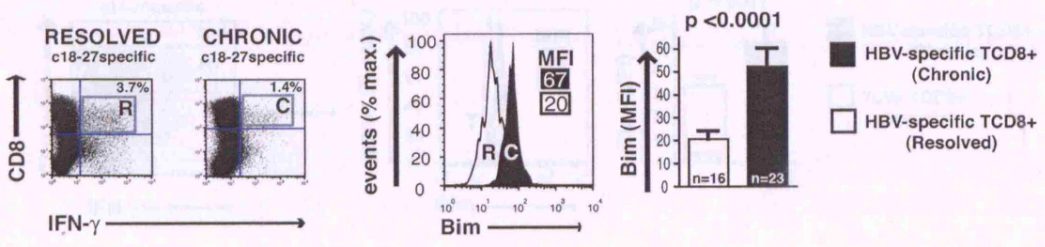

Figure 5.3
Bim expression is increased at the protein level in HBV-specific CD8+ T cells from patients with CHB.
Representative example of Bim expression in HBV-specific CD8+ T cells in individuals with resolved and chronic infection (left and middle) and the cumulative data, significance tested with the Mann Whitney test (right).

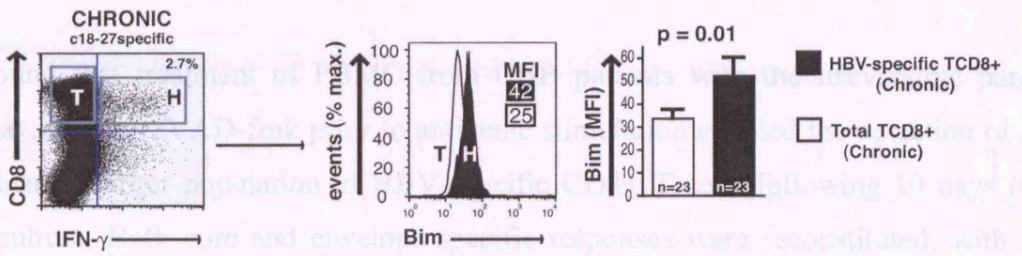

Figure 5.4
Bim expression at the protein level in HBV-specific CD8+ T cells compared to total CD8+ T cells within the same CHB patients.
Representative example of Bim expression in total CD8+ T cells (T) and HBV-specific CD8+ T cells (H) in a patient with chronic infection (left and middle) and cumulative data (right)

5.2.3 *Enhanced recovery of HBV-specific CD8+ T cells upon inhibition of apoptotic pathways in vitro*

Bim is able to exert anti-apoptotic activity by inducing dimerization of the multidomain proapoptoic protein Bax (Marani et al., 2002; Weber et al., 2007) allowing release of cytochrome c that in turn activates caspases that drive cell death (Sehra and Dent, 2006). By artificially interfering with intracellular caspase activity, we attempted to block the intrinsic death-signalling cascade and therefore rescue HBV-specific CD8+ T cells that had upregulated Bim and were otherwise destined to die.

We found that treatment of PBMC from CHB patients with the irreversible pan-caspase inhibitor zVAD-fmk prior to antigenic stimulation enabled the detection of a significantly larger population of HBV-specific CD8+ T cells following 10 days *in vitro* culture. Both core and envelope-specific responses were reconstituted, with a mean three fold increase in virus-specific numbers compared to cells that were stimulated without the inhibitor (figure 5.5 A&B; $p<0.0001$; paired t test).

We also found that responses to additional HBV envelope epitopes could occasionally be recovered (data not shown), demonstrating that this approach could potentially enhance the multispecificity of the HBV response. The percent of total CD8+ T cells did not increase in these experiments ($p=0.48$; paired t test), indicating that this rescue was restricted to the HBV-specific populations (figure 5.6).

We also examined influenza-specific CD8+ T cell responses in the same persistently HBV-infected patients and found that these did not increase following caspase inhibition (Figure 5.7) demonstrating that Bim-mediated deletion of lymphocytes was restricted to the HBV-specific CD8+ T cell compartment and was not a generalized occurrence phenomenon of all virus-specific cells; influenza A virus, was efficiently controlled by the host immune response in the CHB infected patients studied.

Furthermore, HBV-specific CD8+ T cell responses expanded from patients who had resolved infection were also not prone to caspase-dependent apoptosis *in vitro*, as evidenced by the inability to increase anti-viral frequencies following treatment with the inhibitor (Figure 5.8). This supported the fact that deletion was predominantly restricted to HBV-specific CD8+ T cells associated with persistent infection.

Caspase activation can also be triggered by extrinsic mechanisms through cell surface death receptors, therefore, inhibition of caspases was not exclusive proof of the involvement of this pro-apoptotic protein in the deletion of virus-specific CD8+ T cells. The lack of a direct Bim inhibitor complicated these investigations, however, we were able to utilize a pentapeptide (VPLMK) that inhibits the proapoptotic mediator Bax by suppressing its mitochondrial translocation (Sawada et al., 2003a; Sawada et al., 2003b). In this way, we could disrupt the death-signalling cascade upstream of caspases but directly downstream of Bim. We found that this inhibitor was also capable of enhancing the recovery of HBV-specific responses in culture (Figure 5.9) and provided further support for the activity of Bim in the deletion of these antiviral populations.

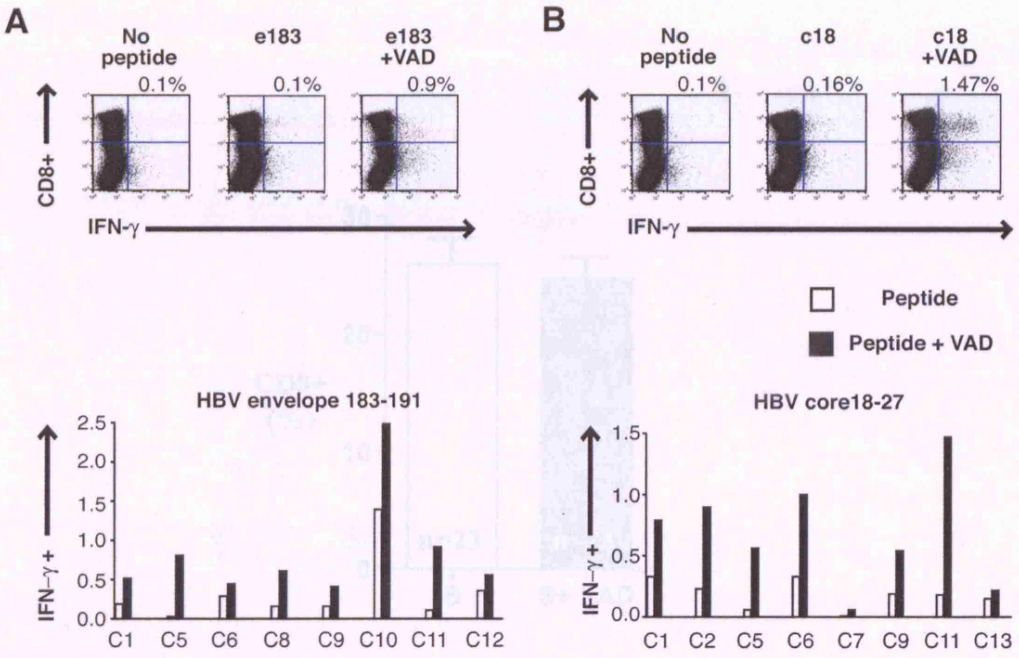

Figure 5.5
Rescue of *in vitro* cultured HBV-specific CD8+ T cells.
Representative flow cytometry plots and cumulative data (below) of the effect of pan-caspase inhibition on the detection of envelope and core-specific CD8+ T cells (A & B) in 10 day lines of PBMCs from individuals with chronic infection.

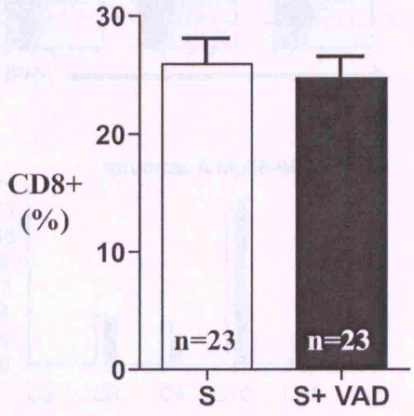

Figure 5.6
Frequency of total CD8+ T cells.
Percent of CD8+ T lymphocytes in peptide-stimulated short-term enriched lines from individuals with chronic infection either untreated or treated with the pan caspase inhibitor (zVADfmk).

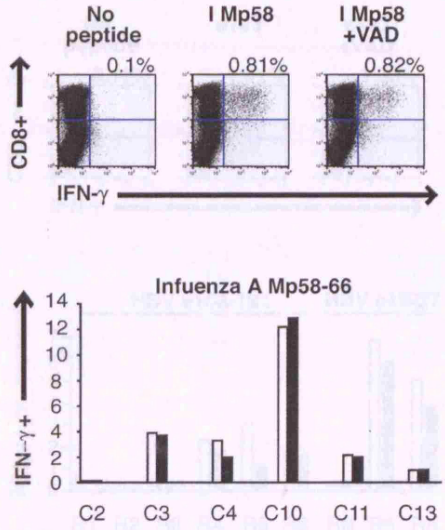

Figure 5.7
Effect of caspase inhibition on influenza-specific CD8+ T cells in patients with chronic HBV infection.
Representative flow cytometry plots and cumulative data (below) of the effect of pan-caspase inhibition on the detection of influenza A-specific CD8+ T cells in 10 day lines of PBMCs from individuals with chronic infection.

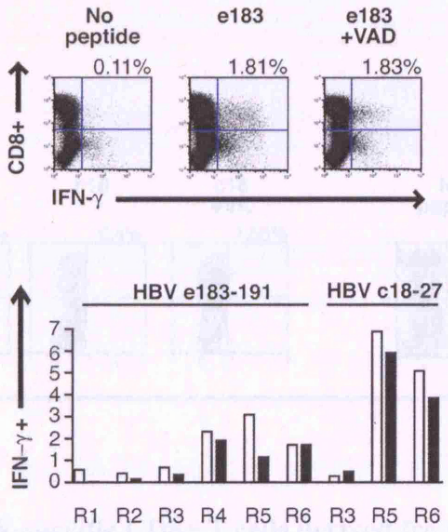

Figure 5.8
Effect of caspase inhibition on HBV-specific CD8+ T cells in patients with resolved HBV infection
Representative flow cytometry plots and cumulative data (below) of the effect of pan-caspase inhibition on the detection of envelope and core-specific CD8+ T cells in 10 day lines of PBMCs from individuals with resolved HBV infection.

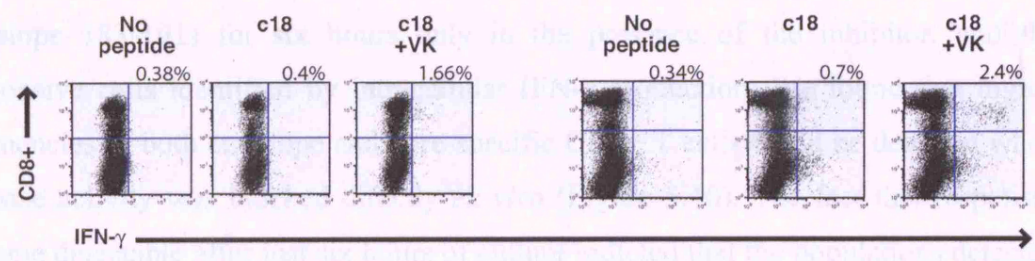

Figure 5.9
Rescue of HBV-specific CD8+ T cells derived from individuals with CHB by inhibition of Bax.
HBV-specific CD8+ T cells rescue in two patients with chronic infection following in vitro culture with a specific peptide inhibitor (VK) of proapoptotic Bax .

5.2.4 Direct ex vivo rescue of HBV-specific CD8+ T cells from patients with CHB

The experiments on short-term enriched cell lines provided functional confirmation of the dysregulated apoptotic pathways identified by microarray profiling. They confirmed that HBV-specific CD8+ T cells from patients with CHB infection are highly susceptible to apoptosis following restimulation with cognate peptide *in vitro*. In order to determine whether these populations were also apoptosis-prone when circulating in patients with high viral load, we studied the effect of caspase-inhibition directly *ex vivo*.

PBMC from the same patients were stimulated with HBV peptide (core18-27 or envelope 183-191) for six hours only in the presence of the inhibitor, and the responsive cells identified by intracellular IFN-γ production. We found that higher frequencies of both envelope and core-specific CD8+ T cells could be detected when caspase activity was blocked directly *ex vivo* (Figure 5.10). The fact that responses became detectable after just six hours of culture indicted that the populations detected were a consequence of the inhibition of apoptosis rather than due proliferation.

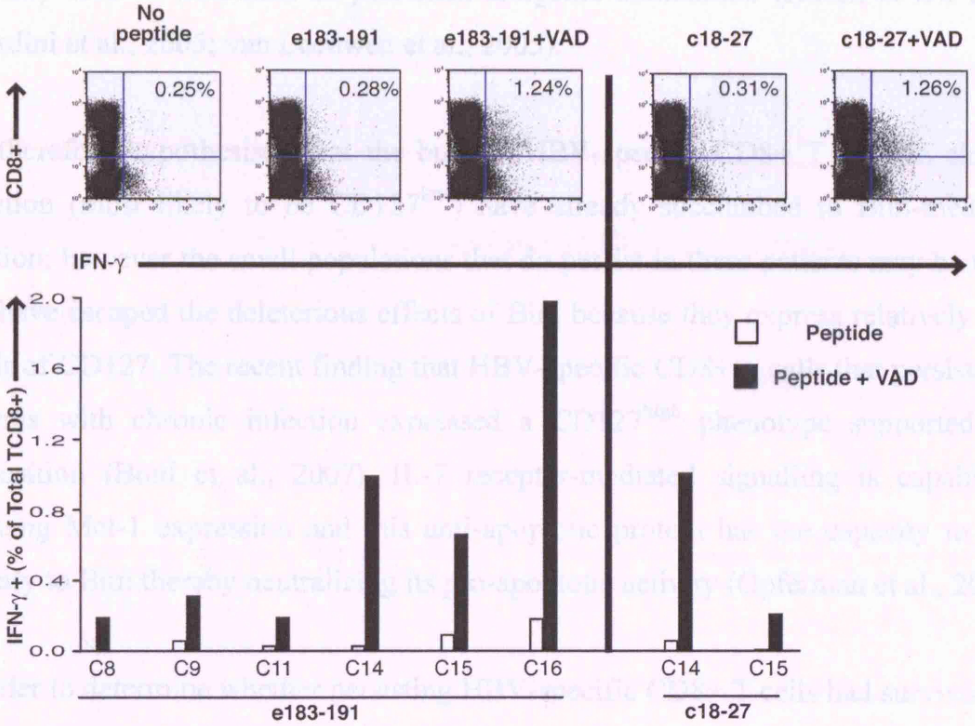

Figure 5.10

Direct *ex vivo* rescue of HBV-specific CD8+ T cells from patients with CHB.
Representative flow cytometry plots and cumulative data (below) indicating direct *ex vivo* frequencies of HBV-specific CD8+ T cells in patients with chronic infection as detected following stimulation with viral peptide, with and without treatment with the pan-caspase inhibitor zVAD-fmk.

5.2.5 *HBV-specific CD8+ T cells persisting in the face of high antigen load are selectively enriched for high expression of CD127 and Mcl-1*

Studies have shown that the CD8+ T cell populations that are killed through Bim-mediated mechanisms are those that express low levels of the IL-7 receptor α chain (CD127) (Pellegrini et al., 2004; Wojciechowski et al., 2006). This phenotype is typically seen in situations of persistent antigenic stimulation (Kaech et al., 2003; Paiardini et al., 2005; van Leeuwen et al., 2005).

We therefore hypothesised that the bulk of HBV-specific CD8+ T cells in chronic infection (most likely to be $CD127^{low}$) have already succumbed to Bim-mediated deletion; however the small populations that do persist in these patients may be those that have escaped the deleterious effects of Bim because they express relatively high levels of CD127. The recent finding that HBV-specific CD8+ T cells that persisted in patients with chronic infection expressed a $CD127^{high}$ phenotype supported this proposition (Boni et al., 2007). IL-7 receptor-mediated signalling is capable of inducing Mcl-1 expression and this anti-apoptotic protein has the capacity to bind directly to Bim thereby neutralizing its pro-apoptotic activity (Opferman et al., 2003).

In order to determine whether persisting HBV-specific CD8+ T cells had survived the effects of increased Bim though the upregulation of Mcl-1, we measured intracellular levels of this latter protein. We found that Mcl-1 levels were indeed strikingly elevated in the core and envelope-specific CD8+ T cells, with an MFI approximately double that observed in CD8+ T cells of the same specificity from patients who had resolved their HBV infection (figure 5.11 A&B). Mcl-1 levels were also significantly higher ($p=0.0001$) in HBV-specific (IFN-γ positive) than total CD8+ T cells (IFN-γ negative) within the same patients, whereas levels in influenza-specific CD8+ T cells were similar to that in total CD8+ T cells (figure 5.11 C). We confirmed that HBV-specific CD8+ T cells in patients with chronic HBV infection were $CD127^{high}$, in accordance to the published data (Boni et al., 2007). By co-staining these populations for CD127 and Mcl-1, we found that all the $CD127^{high}$ HBV-specific CD8+ T cells

also expressed high intracellular levels of Mcl-1, consistent with their rescue through this mechanism (figure 5.11 D).

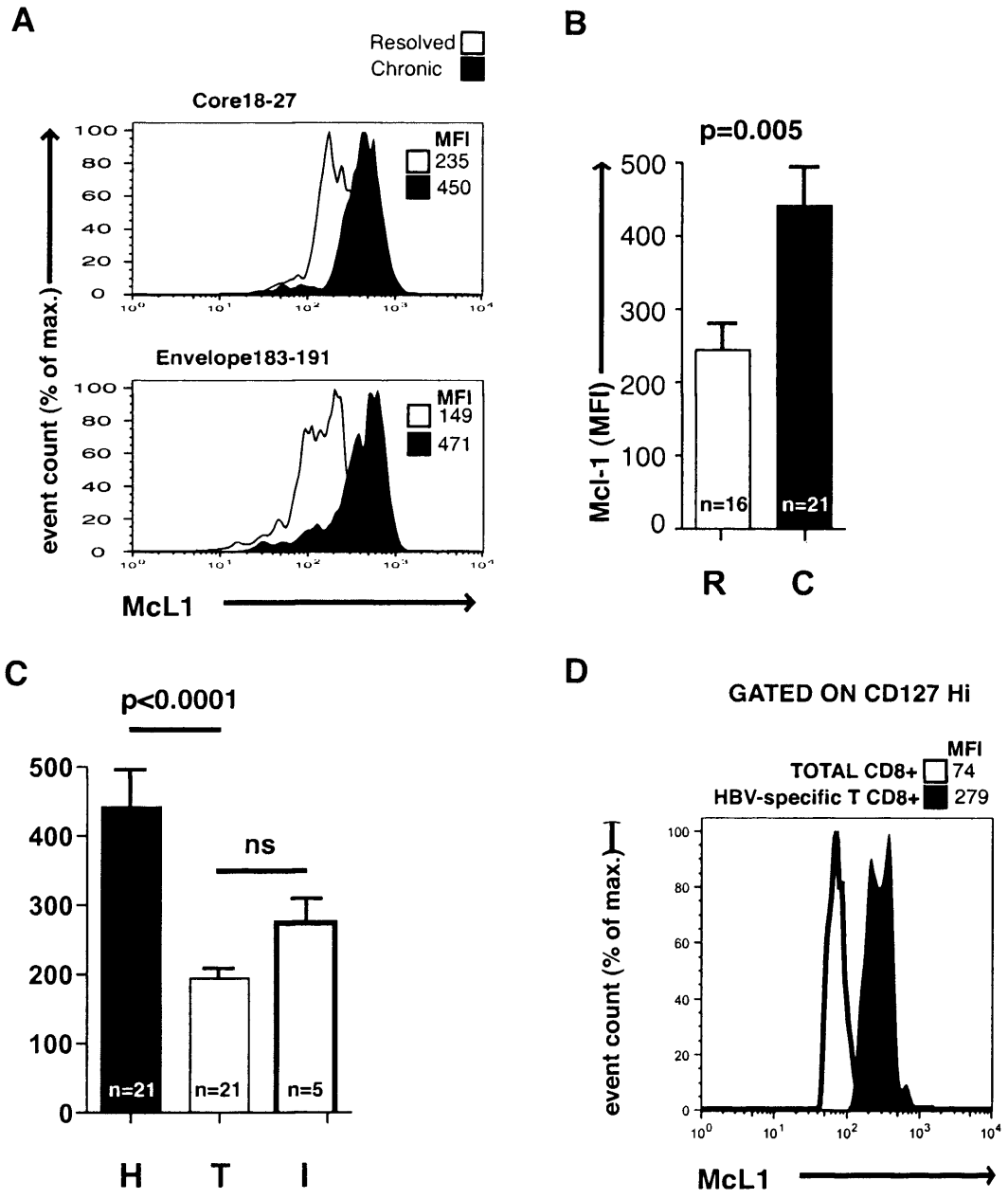

Figure 5.11
Mcl-1 and CD127 expression of HBV-specific CD8+ T cells.
(A) Intracellular staining for Mcl-1 in core and envelope-specific CD8+ T cells from individuals with chronic and resolved infection (A) with cumulative data (B). (C) Summary of Mcl-1 levels in HBV-specific (H), total (T) and influenza (I)-specific CD8+ T cells. (D) Intracellular stain for Mcl-1 in CD127hi populations of HBV-specific and total CD8+ T cells.

5.2.6 *Bim expression in HBV-specific CD8+ T cell populations following cross-presentation of HBV antigens*

Adoptive transfer of CD8+ T cells from influenza hemagglutinin-specific TCR transgenic mice into mice expressing hemagglutinin has been shown to result in tolerance following cross-presentaton of antigen by pAPCs in the draining lymph nodes (Hernandez et al., 2001); deletion of CD8+ T cells was preceeded by an upregulation of CD69 and CD44, partial downregulation of CD62L, the failure to express CD25, the inability to produce IFN-γ, and a lack of cytolytic function. A subsequent study found that cross-presentation of soluble ovalbumin (OVA) in transgenic mice also resulted in the peripheral deletion of the OVA-specific CD8+ T cells (Kurts et al., 1997), but importantly, these populations persisted when T cells were Bim-deficient (Davey et al., 2002). These data indicated that cross-presentation of soluble antigen could drive the deletion of reactive CD8+ T cells through a mechanism involving Bim.

Given that chronic HBV infection is associated with the secretion of large quantities of viral antigens, we hypothesized that HBV-specific CD8+ T cells in chronic infection could also be prone to Bim-mediated tolerance following cross-presentation of these soluble HBV antigens.

In order to test this hypothesis *in vitro*, we employed the well-characterised HLA-A2+ MUTZ-3 monocytic cell line (kindly provided by B.Chain, U.C.L) that can be differentiated into professional antigen presenting cells (Larsson et al., 2006; Masterson et al., 2002; Santegoets et al., 2006); exposure to IL-4 and GM-CSF induces differentiation into a dendritic cell-like populations that are able to present antigen to CD8+ T cells (Santegoets et al., 2006). We aimed to use MUTZ-3-derived DCs (M-DC) to cross present HBV antigens to HBV-specific CD8+ T cells that had relatively low levels of Bim and then evaluate the effect that this interaction had on the expression of Bim in these antiviral CD8+ T cell populations.

To achieve this, total PBMCs from three individuals that had resolved infection were enriched for HBV-specific CD8+ T cells by stimulating cells with immunodominant viral peptides and allowing them to proliferate over 10 days, as done previously. We have already demonstrated that these enriched virus-specific populations had relatively low levels of Bim compared to HBV-specific CD8+ T cells from individuals with chronic infection.

At day 10, the enriched cell lines were restimulated (for 5 hours) either with HBV peptides alone or M-DC that had been previously loaded with hepatitis B surface and core antigen. The frequency of HBV-specific CD8+ T cells (IFN-γ+ by ICS) and the quantity of Bim in these populations were then determined. HBV-specific CD8+ T cells in enriched lines stimulated with peptide alone are primed either by endogenous APCs or by fraternal CD8+ T cells that express empty cell surface MHCI (unpublished observations by A.Bertoletti, Singapore Institute of Clinical Sciences). This M-DC-independent stimulation was used to assess the HBV-specific CD8+ T cel frequency following enrichment for each patient sample as well as to allow us to bench-mark the quantity of Bim in cells that were not stimulated through cross-presentation.

5.2.6.1 Mutz-derived dendritic cells cross-present soluble HBV antigens to HBV-specific CD8+ T cells

Resolved patients 1, 2 and 3 (R1, R2, R3) had variable HBV-specific CD8+ T cell populations at day 10 (15.9, 1.54 and 1.27% of total CD8+ T respectively) (figure 5.12), as detected by peptide restimulation. We found that HBsAg-loaded M-DC could induce HBV-specific T cells to produce IFN-γ in all three patients (3.09, 1.17 and 2.8% for R1, R2 and R3 respectively) (Figure 5.12). Although enriched lines stimulated with unloaded M-DCs resulted in some non-specific IFN-γ production that was slightly above the unstimulated controls, this was well below the frequencies that were detected with the antigen loaded M-DC (Figure 5.12; R2). These data thus confirmed that *in vitro* generated pAPC were able to take up soluble antigen, digest,

load and cross-present critical antigenic determinants via MHCI to HBV-specific CD8+ T cells.

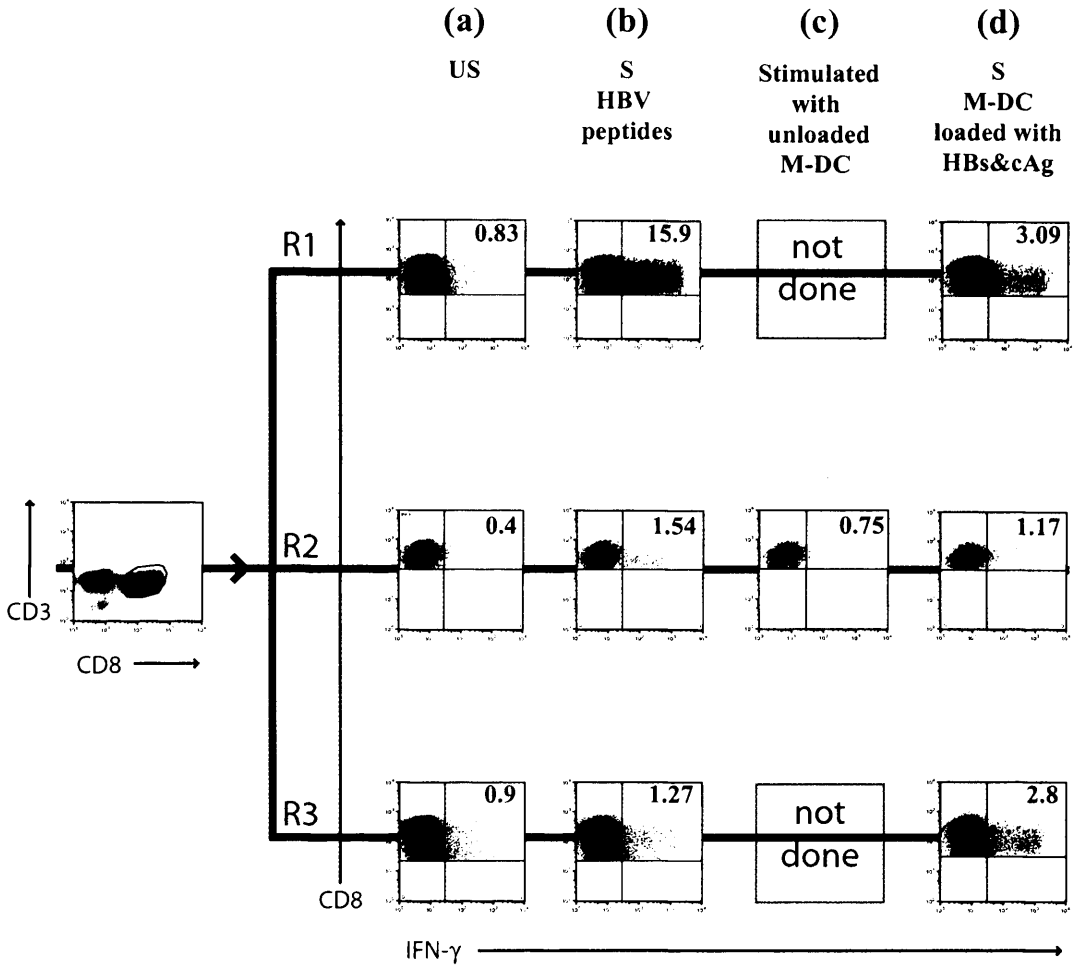

Figure 5.12
***In vitro* stimulation of CD8+ T cells with antigen-loaded Mutz-3-derived dendritic cells.**
HBV-specific CD8+ T cell were enriched in PBMCs from three patients (R1, R2 and R3) that resolved infection by stimulation with a pool of HBV peptides (c18-27, e183-191, e335-343, e338-347, e348-357, p455-463, p502-510) followed by 10 days of culture. At day 10, appropriate samples were either unstimulated (a), restimulated with HBV peptides (b), restimulated with unloaded M-DCs (c), or restimulated with M-DCs loaded with HBs&cAg (d).

5.2.6.2 Bim expression

In line with the previous data (figure 5.3; where the average of the median fluorescence intensity for Bim in HBV-specific CD8+ T cells from resolved individuals was 20.7; n=23), the level of Bim in peptide alone-stimulated HBV-specific CD8+ T cells in R1, R2 and R3 was also correspondingly low (MFI of 21.4, 28.4 and 24.9; mean of 24.9).

In samples R1 and R2, HBV-specific CD8+ T cells stimulated HBs&cAg-loaded M-DC (compared to peptide stimulated samples) exhibited slightly raised levels of Bim (MFI of 23 versus 21.4 and 34.9 versus 28.4 respectively). (figure 5.13, top and middle graphs). The exception however was R3 (figure 5.13, bottom graph). Here a slight decline in Bim was observed in the HBV-specific CD8+ T cell population following stimulation with HBs&cAg loaded M-DC compared to the peptide alone stimulation (Bim MFI=21.4 and 24.9 respectively).

These differences are not strikingly different, however, these preliminary studies require further optimization, particularly with regard to the duration of stimulation. It is likely that five hours, as applied in this study, may not be sufficient to induce a significant difference in the levels of Bim expression.

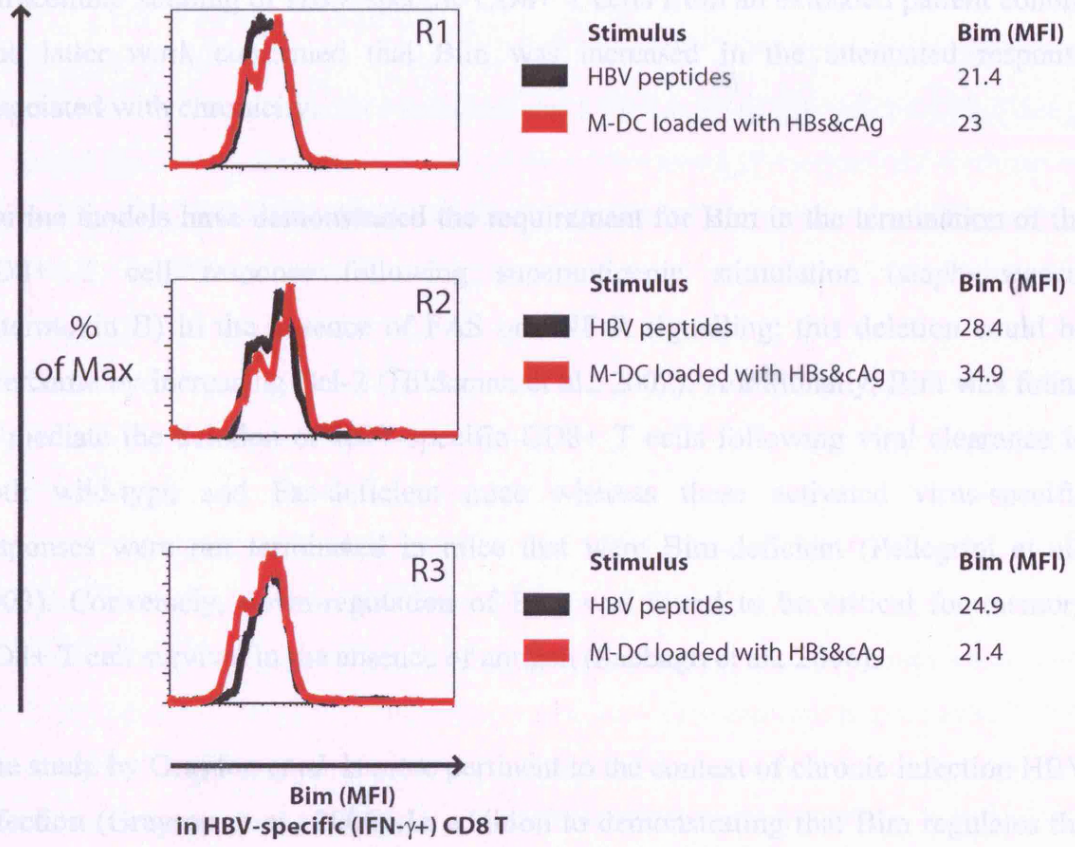

Figure 5.13
Bim expression in HBV-specific CD8+ T cells following stimulation by APCs cross-presenting HBc&sAg.
HBV-specific CD8+ T cells were enriched in PBMCs from three patients that resolved infection by stimulation with a pool of HBV peptides (c18-27, e183-191, e335-343, e338-347, e348-357, p455-463, p502-510) followed by 10 days of culture. Enriched PBMCs were restimulated either with HBV peptides (black) or with M-DC (red) loaded with whole HBV protein (core and envelope). Intracellular levels of Bim (median fluorescence intensity) was evaluated in HBV-specific (IFN-γ+) CD8+ T cells by flow cytometry.

5.3 Discussion

In the previous chapter, cDNA microarray analysis revealed that the most consistently up-regulated gene in HBV-specific CD8+ T cells in chronic infection with strongest statistical significance was the Bcl-2 interacting mediator (Bim). Here, we validated these data at the transcriptional level by demonstrating similar results with real-time QPCR and subsequently extended this to the protein level by intracellular staining of HBV-specific CD8+ T cells from an extended patient cohort. The latter work confirmed that Bim was increased in the attenuated response associated with chronicity.

Murine models have demonstrated the requirement for Bim in the termination of the CD8+ T cell response following superantigenic stimulation (staphylococcal enterotoxin B) in the absence of FAS or TNF-R signalling; this deletion could be overcome by increasing Bcl-2 (Hildeman et al., 2002). Additionally, Bim was found to mediate the deletion of HSV-specific CD8+ T cells following viral clearance in both wild-type and Fas-deficient mice whereas these activated virus-specific responses were not terminated in mice that were Bim-deficient (Pellegrini et al., 2003). Conversely, down-regulation of Bim was found to be critical for memory CD8+ T cell survival in the absence of antigen (Sabbagh et al., 2006).

The study by Grayson *et al.* is more pertinent to the context of chronic infection HBV infection (Grayson et al., 2006). In addition to demonstrating that Bim regulates the CD8+ T cell response during chronic LCMV infection in mice, they have also shown that this loss was particularly focussed on the immunodominant LCMV-specific CD8+ T cell response, which parallels chronic HBV infection where responses to an immunodominant core epitope become undetectable in patients with high viral loads (Webster et al., 2004).

Bim mediates the apoptosis of CD8+ T cells that have low levels of the IL-7 receptor alpha chain (CD127) (Wojciechowski et al., 2006) and is also a major apoptotic factor in virus-specific CD8+ T cells that bear this phenotype (Pellegrini et al., 2004).

In agreement with these data, we found that the HBV-specific CD8+ T cells that managed to survive in chronic HBV infection maintained expression of CD127. The maintenance of CD8+ T cells expressing high levels of CD127 has been described in chronic HBV (Boni et al., 2007) and HCV (Bengsch et al., 2007; Penna et al., 2007; Radziewicz et al., 2007) infection but contrasts to chronic HIV, CMV and EBV infections (Paiardini et al., 2005; van Leeuwen et al., 2005) where low levels were observed.

We propose that the majority of HBV-specific CD8+ T cells expressing low levels of CD127 have succumbed to Bim-mediated deletion but those populations that manage to persist have neutralized this proapoptotic-factor through mechanisms initiated by rescue signals delivered by IL-7 through its cognate receptor (Kaech et al., 2003). In particular, IL-7/IL-7R signalling triggers an increase in the anti-apoptotic protein Mcl-1 (Opferman et al., 2003) and this protein has been shown to play an essential role in mature lymphocyte survival by counteracting the pro-apoptotic effects of Bim (Opferman et al., 2003). In line with these findings we found that the persisting HBV-specific CD8+ T cells had raised levels of Mcl-1.

The obvious conclusion is that these small populations persist by because of their ability to counteract Bim-mediated deletion. Alternatively, recent data has shown that newly generated T cells are continuously recruited to the antiviral response in chronic LCMV infection (Vezys et al., 2006); it is therefore possible that the expression of CD127 reflects a recently primed status of the HBV-specific CD8+ T cells. This implies that that the HBV-specific CD8+ T cell response in chronically infected individuals has a higher turnover than previously realised. It also suggests that the protection by Mcl-1 is only temporary; continual recruitment would result in an increasing frequency of surviving cells. Alternatively, competitive scavenging for limited quantities of IL-7 would only allow some populations to persist.

By interfering with Bim-mediated apoptosis through the inactivation of caspases and pro-apoptotic Bax, we were able to rescue HBV-specific CD8+ T cells in culture. Apart from providing functional confirmation of the microarray data and supporting

the need to further investigate other apoptosis-related short-listed genes, this highlighted a strategy that could potentially be used to recover these populations.

Disrupting PD-1/PD-L1 interactions has recently been found to reverse virus-specific CD8+ T cell dysfunction in patients with CHB infection (Boni et al., 2007) but only of certain specificities; envelope-specific responses could not be detected, suggesting that these were prone to an alternative tolerogenic mechanism that is very likely to be related to the excessive quantities of surface antigen produced by those patients. We have shown that blockade of Bim-mediated apoptosis allows the recovery of both core and envelope specificities. Furthermore, inhibition of this apoptosis pathway directly *ex vivo* also resulted in substantial rescue of HBV-specific CD8+ T cells, indicating that circulating responses are highly susceptible to apoptosis in patients with chronic infection.

These data do not argue against the involvement of negative co-stimulatory ligands and receptors such as PD-1. Instead, we propose that in chronic infection, it is likely that the tolerogenic signals have already been delivered to antigen-specific CD8+ T cells, and it would be more beneficial to interfere with the downstream events that lead to deletion of these populations. In this regard, we would only expect to be able to achieve a limited amount of reconstitution *ex vivo* as most antiviral populations would have been deleted.

The transient restoration of HBV-specific responses reconstituted during antiviral therapy (Boni et al., 2003) suggests that a short-term reduction in viral load is not sufficient to reverse their propensity to apoptosis. As activation of protein kinase C and calcineurin is necessary for Bim up-regulation following TCR ligation (Sandalova et al., 2004), a strategy aimed at blocking Bim induction (with Cyclosporin A or FK506) could be more effective at protecting recent thymic emigrants capable of recognising the virus. However, these studies must also take into account the confounding generalized immunosupproessive activity of these agents.

Thus, in addition to preventing apoptosis in HBV-specific CD8+ T cells with upregulated Bim, specific re-programming of HBV-specific CD8+ T cell susceptibility to Bim-mediated apoptosis in patients with CHB infection (undergoing suitable antiviral therapy) holds greater potential for the reconstitution of effective HBV-specific responses.

The mechanism specifically driving the upregulation of Bim in HBV-specific CD8+ T cells in CHB remains to be identified. It may well be the level of antigenic drive and activation status of responding cells, since T cell receptor triggering has been shown to induce Bim in effector CD8+ T cells (Sandalova et al., 2006; Sandalova et al., 2004). This is consistent with the preferential deletion of immunodominant responses (Grayson et al., 2006) and is in keeping with our data, which focused on two frequently recognised HBV epitopes (core 18-27 and envelope 183-191) from antigens that are produced at high concentrations in this infection.

It will be important to investigate whether Bim levels are lower in the subdominant responses present in chronic infection, but these studies can only be executed once the hierarchy of HBV-specific CD8+ T cell responses restricted by diverse HLA alleles has been better defined. Preliminary data indicates that the level of Bim in a polymerase-specific response is lower than that in core and envelope-specific CD8+ T cells in CHB in line with the fact that polymerase is expressed at a much lower quantities compared to the surface and core proteins.

Another possibility could be the actual site of CD8+ T cell priming. Studies in transgenic mouse models have shown that antigen that is endogenously processed and presented by hepatocytes induces proliferation that is followed by anergy or deletion of the responding CD8+ T cells (Bertolino et al., 1998; Morimoto et al., 2007). Human HBV-specific CD8+ T cells that recognise antigen presented by HBV-infected human hepatocytes (Gehring et al., 2007) also become highly prone to apoptosis (unpublished observations; A. Bertoletti et al, Singapore Institute of Clinical Sciences); the involvement of Bim in this process remains to be investigated.

An alternative factor could be related to the large amounts of soluble surface and HBeAg are produced and secreted by hepatocyes in CHB infection as these antigens could access the MHCI processing pathway for cross-presentation (Jin et al., 1988). Liver sinusoidal endothelial cells or hepatic stellate cells (Winau et al., 2007), are particularly well positioned to take up exogenous soluble antigen released from infected hepatocytes, and have been shown to induce cross-tolerance of Ovalbumin-specific CD8+ T cells in transgenic mice (Limmer et al., 2000). Cross-presentation of antigens released from apoptotic cells has also been associated with induction of tolerance in the liver (Albert et al., 1998; Berg et al., 2006).

Our preliminary studies aimed at examining tolerance through cross-priming demonstrated that pAPCs could effectively present whole soluble HBV antigens to HBV-specific CD8+ T cells but we did not detect significant increases in the expression of Bim following activation by protein loaded M-DCs. This could be related to the duration of stimulation of our assay. Although two hours of contact are sufficient to initiate cell division, approximately 24 hours of contact is required for full programming (Kaech and Ahmed, 2001; Kaech et al., 2002; van Stipdonk et al., 2001); the deletion of CD8+ T cells is not programmed by a brief interaction with tolerogenic APCs but has been shown to rely on persistent antigenic stimulation that could act by maintaining the tolerising signals for long enough to overcome survival signals delivered by certain cytokines (Redmond et al., 2003). Our assay employed a five-hour stimulation that may not have been sufficient to detect significant changes in Bim expression; further studies should be aimed at addressing this factor.

However, in addition to the strength of signalling at the level of individual TCRs, growing evidence suggests that the maturation state of professional APCs is critical in determining whether the CD8+ that they prime are instructed to undergo a vigorous effector response or anergy and death. Multiple signals including triggers from the pathogen itself, proinflammatory cytokines or CD4+ T cell help, lead to an upregulation of costimulatory molecules (Banchereau and Steinman, 1998; Bennett et al., 1998; Ridge et al., 1998; Schoenberger et al., 1998; Steinman et al., 1997), also known as licensing (Marsland et al., 2005; Smith et al., 2004), that is required for

optimal CD8+ T cell priming (Larsson et al., 2000; Salio et al., 2001) (Bachmann et al., 1999b), in fact, the efficacy of vaccines is significantly improved when administered with some form of adjuvant (Bendelac and Medzhitov, 2002; Cooper et al., 2005; Cooper et al., 2004); this is most likely due to the initiation of a local inflammatory reaction that drives maturation of pAPCs that take-up the foreign antigen that is key to the priming of an effective T cell response.

Although a small proportion of T cells may survive priming by unlicensed resting DCs in draining lymph nodes (van Mierlo et al., 2002), the absence of costimulation leads to the premature death of most T cells (Hawiger et al., 2001; Kaech and Ahmed, 2001; Kaech et al., 2002; Sauter et al., 2000; van Stipdonk et al., 2001).

Thus, the maturation state of the M-DCs appears to be of particular importance. LPS treatment can drive maturation of M-DCs as evidenced by increases co-stimulatory molecules (CD80, CD86, CD40) and the activation marker HLA-DR (personal communication; B.Chain, U.C.L.). Although the M-DCs utilized in this study were immature, it is possible that traces of LPS in the whole HBV antigen preparations were of sufficient quantity to trigger some degree of maturation that could have influenced Bim expression.

Studies have shown that anti-inflammatory glucocorticoids, Dexamethasone in particular, can be used to maintain immature DCs with tolerogenic potential despite stimulation with conventional maturation stimuli (Matyszak et al., 2000; Moser et al., 1995; Piemonti et al., 1999; Rea et al., 2000; Roelen et al., 2003; Woltman et al., 2000; Xia et al., 2005). This enables us to manipulate the maturation status of the APCs in future studies in order to assess its importance to the process of cross-tolerance.

In conclusion, the profound HBV-specific CD8+ T cell hyporesponsiveness that is observed in CHB infection could be due to mechanisms related to the high level of antigen load in these patients. In this study, a global, unbiased approach allowed us to dissect these mechanisms and highlighted a dysregulated apoptotic pathway. Preliminary studies suggest that cross-presentation of HBV antigens and subsequent Bim-mediated deletion could contribute to the failure of CD8+ T cell responses in chronic HBV infection. Interfering with this tolerogenic mechanism may provide a new strategy to sustain the HBV-specific responses that are indispensable for effective viral control.

6 *Future directions*

Our data indicate that the immunodominant CD8+ T cell response associated with resolution of infection is prone to deletion in chronic infection. However, these studies were focused on a limited number of epitopes and it is critical that future studies aim to generate more comprehensive profiles of HBV-specific CD8+ T immunodominance hierarchies and their association with immune failure.

Overlapping peptides that span all viral proteins could be used in conjunction with flow cytometry and elispot assays to obtain a more representative definition of the overall immune response to HBV (Boni et al., 2007; Chang et al., 2005). These should match the sequence of the infecting virus but as this is currently unrealistic, efforts should at least be made to utilize a peptide panel that corresponds to the viral genotype involved. These studies should ideally be conducted directly *ex vivo* but responses are rarely detectable when studied in this way (Boni et al., 2007; Chang et al., 2005) therefore the use of short-term *in vitro* enrichment must continue in order to detect low-level frequencies.

Once key responses have been identified, there are various strategies that may be applied to enhance these frequencies for further study:

1) selective enrichment based on effector function such as IFN-γ could be used to increase populations of virus-specific CD8+ T cells normally present at very low levels as has been described (Barnes et al., 2004).

2) endogenous (T or B) regulatory cells are able to exert generalised (non-contact dependent) and specific (contact-dependent) immunosuppressive activity (Accapezzato et al., 2004; Boussiotis et al., 2000b; Hyodo et al., 2004; Plebanski et al., 1999; Pohl-Koppe et al., 1999). Depleting these populations could help increase the HBV-specific CD8+ T cell frequency. Recent data suggest that virus-specific populations may themselves produce and secrete immuosuppressive mediators such as IL-10 (Abel et al., 2006; Elrefaei et al., 2007). Activity could be blocked with anti-

IL-10 antibodies (Maris et al., 2007), or alternatively, antagonistic anti-IL-10 receptor antibodies could be used to interfere with immune suppression (Brooks et al., 2006b) (Ejrnaes et al., 2006).

3) Data from our microarray analyses indicate that TGF-β is also up-regulated in HBV-specific CD8+ T cells associated with chronic infection. Several studies have implicated this cytokine in immune suppression (Alatrakchi et al., 2007; Garba et al., 2002; Kekow et al., 1990; Lotz et al., 1990) and anti-TGF-β strategies similar to those proposed for the neutralization of IL-10 activity could be investigated to see if they improve the detection of HBV-specific antiviral CD8+ T cell populations.

Returning to the findings of the cDNA microarray analyses, we found that the core and envelope specificities that were most prevalent in resolved infection had elevated levels of proapoptotic factors, particularly Bim, during persistent HBV infection, consistent with studies that demonstrate the selective deletion of T cells specific for antigen present at a high level (Wherry et al., 2003) (Grayson et al., 2006). We propose that this could be due to tolerance mechanisms involving cross-presentation of soluble HBeAg and HBsAg as reported recently (Davey et al., 2002). Studies of Bim expression in HBV polymerase- and X-specific CD8+ T cell could help provide a better understanding of the relationship between cross-presentation, tolerance and exhaustion, given that the polymerase and X protein are not secreted are present in comparatively reduced quantities.

Ongoing studies are aimed at establishing an *in vitro* model to investigate the mechanism of cross-tolerance of HBV-specific CD8+ T cells. As memory CD8+ T cells are susceptible to peripheral deletion following secondary encounter with antigen (Kreuwel et al., 2002), functional HBV-specific CD8+ T from resolved individuals could help further understand the specific mechanism of tolerance involved.

One important consideration in these studies is that commercially produced HBsAg and HBcAg is produced in bacterial systems and will be prone to lipopolysacchride

contamination that will undoubtedly interfere with the assay employed. Given that the maturation status of the antigen presenting cells may be the key factor in the process of tolerance, and that LPS mediates maturation, this issue needs to be resolved. This will involve characterisation of the maturation status of the antigen presenting cells prior to and following loading with the HBcAg and HBsAg. We also aim to test the use of the dexamethasone in overcoming this problem; this steroid has been successfully used to maintain immaturity of pAPCs (Xia et al., 2005).

The data arising from this work indicate that interfering with Bim-mediated deletion of HBV-specific CD8+ T cells may facilitate successful containment of the pathogen in individuals with chronic infection. This can be readily translated to the clinical setting because calcineurin or protein kinase C inhibitors (tacrolimus/cyclosporin or tamoxifen respectively) that can inhibit Bim upregulation (Sandalova et al., 2004) are already in clinical use. Treatment of HBV infected individuals was in fact associated with a slight increase of the HBV-specific CD8+ T cell response during but not after steroid (prednisolone) therapy (Gotto et al., 2006).

Our data also indicates that inhibition of Bax, the death mediator further downstream of Bim, can also rescue antiviral specificities. Given that the proapoptotic cascade involves multiple death mediators, there are in fact multiple specific factors, in addition to Bim, that could potentially be targeted and this may be particularly important for the rescue of cells that have already upregulated Bim.

The overall strategy should therefore aim to combine antivirals with some form of immune rescue that we suggest could be achieved by blocking Bim. Although steroid therapy is normally administered for immunosuppressive purposes, the influence of these agents on antigen-specific CD8+ T cells may depend on their state of activation or differentiation (Ashwell et al., 2000; Riccardi et al., 2002). Nonetheless, initial studies could aim employ short courses of steroids. Additionally, previous studies have demonstrated that immune reconstitution following antiviral therapy was only transient, however, those studies were conducted following short-term therapy with lamivudine, and a more prolonged course of treatment of perhaps three years with

more potent antivirals may be required to confer a more sustained improvement in the CD8+ T cell response. Furthermore, the combination of antiviral drugs therapy with specific apoptotic inhibitors may even facilitate the reprogramming of anergised antiviral T cell populations as has been proposed recently (Brooks et al., 2006a).

Finally, one factor that has been overlooked in HBV studies has been the importance of the route of infection. It is important to consider whether the individuals studied were infected vertically or horizontally as this would have strong implications on the extent of tolerance induced in these individuals. Perinatal transmission would lead to the development of an immune system that considers HBV antigens as self-protein, and therefore, the majority of HBV-reactive CD8+ T cells would be deleted in the thymus.

In these individuals, it might be necessary to reengineer autologous CD8+ T cells to specifically recognize key HBV determinants. We are currently collaborating with Hans Stauss (U.C.L) on a project aimed at transducing CD8+ T cells with a TCR specific for the immunodominant HLA-A2-restricted core 18-27 determinant. Focusing the introduction of this receptor specifically into memory CD8+ T cell populations may overcome costimulatory requirements and therefore generate more effective antiviral populations.

The limitation of this approach would be that the transduced TCR would only be effective in patients with the appropriate HLA-restriction. However, the studies with overlapping peptides proposed at the start of this section will contribute to the development of a database that associates specific HLA-types with commonly targeted viral determinants. These data will contribute to the customization of TCR-transduction technology to suit alternative HLA-types.

7 *Publications arising during the course of this thesis*

Bim-mediated deletion of antigen-specific CD8+ T cells in patients unable to control hepatitis B virus.
Lopes, AR., Kellam. P., Das, A., Dunn, C., Kwan, A., Turner, J., Gilson, R.J., Bertoletti, A., Maini, MK. *Submitted (July 2007)*

A selective reversible defect in the global CD8+ T cell population in chronic hepatitis B virus infection.
Das, A., Hoare, M., Lopes, AR., Dunn, C., Kennedy, PT., Graeme, A., Plunkett, F., Akbar, A., Bertoletti, A., Maini, MK. *Submitted (September 2007)*

Cytokines induced during chronic hepatitis B virus infection promote a pathway for NK cell-mediated liver damage.
Dunn. C., Brunetto. M., Reynolds, G., Christophides, T., Kennedy, PT., Lampertico, P., Das, A., Lopes, AR., Borrow, P., Williams, K., Humphreys, E., Afford, S., Adams, DH., Bertoletti, A, Maini, MK. Journal of Experimental Medicine (2007) 204(3): 667-80

Distinct, cross-reactive epitope specificities of CD8 T cell responses are induced by natural hepatitis B surface antigen variants of different hepatitis B virus genotypes.
Riedl, P., Bertoletti, A., Lopes, R., Lemonnier, F., Reimann, J., Schirmbeck, R. Journal of Immunology (2006) 176(7): 4003-11.

HIV-1 epitope-specific CD8+ T cell responses strongly associated with delayed disease progression cross-recognize epitope variants efficiently.
Turnbull, EL., Lopes, AR., Jones, NA., Cornforth, D., Newton, P., Aldam, D., Pellegrino, P., Turner, J., Williams, I., Wilson, CM., Goepfert, PA., Maini, MK., Borrow P. Journal of Immunology (2006) 176(10): 6130-46.

Effect of HIV infection and antiretroviral therapy on hepatitis B virus (HBV)-specific T cell responses in patients who have resolved HBV infection.
Lascar, RM. & Lopes, AR., Gilson, RJ., Dunn, C., Johnstone, R., Copas, A., Reignat, S., Webster, G., Bertoletti, A., Maini, MK. Journal of Infectious Diseases (2005) 191(7): 1169-79.

Reconstitution of hepatitis B virus (HBV)-specific T cell responses with treatment of human immunodeficiency virus/HBV coinfection.
Lascar, RM., Gilson, RJ., Lopes, AR., Bertoletti, A., Maini, MK. Journal of Infectious Diseases (2003) 188(12): 1815-9.

Greater CD8+ TCR heterogeneity and functional flexibility in HIV-2 compared to HIV-1 infection.
Lopes, AR., Jaye, A., Dorrell, L., Sabally, S., Alabi, A., Jones, NA., Flower, DR., De Groot, A., Newton, P., Lascar, RM., Williams, I., Whittle, H., Bertoletti, A., Borrow, P., Maini, MK. Journal of Immunology (2003) 171(1): 307-16.

8 *Bibliography*

Abdalla, A.O., Kiaii, S., Hansson, L., Rossmann, E.D., Jeddi-Tehrani, M., Shokri, F., Osterborg, A., Mellstedt, H., and Rabbani, H. (2003). Kinetics of cytokine gene expression in human CD4+ and CD8+ T-lymphocyte subsets using quantitative real-time PCR. Scandinavian journal of immunology *58*, 601-606.

Abel, M., Sene, D., Pol, S., Bourliere, M., Poynard, T., Charlotte, F., Cacoub, P., and Caillat-Zucman, S. (2006). Intrahepatic virus-specific IL-10-producing CD8 T cells prevent liver damage during chronic hepatitis C virus infection. Hepatology (Baltimore, Md *44*, 1607-1616.

Accapezzato, D., Francavilla, V., Paroli, M., Casciaro, M., Chircu, L.V., Cividini, A., Abrignani, S., Mondelli, M.U., and Barnaba, V. (2004). Hepatic expansion of a virus-specific regulatory CD8(+) T cell population in chronic hepatitis C virus infection. The Journal of clinical investigation *113*, 963-972.

Accapezzato, D., Visco, V., Francavilla, V., Molette, C., Donato, T., Paroli, M., Mondelli, M.U., Doria, M., Torrisi, M.R., and Barnaba, V. (2005). Chloroquine enhances human CD8+ T cell responses against soluble antigens in vivo. The Journal of experimental medicine *202*, 817-828.

Ackerman, A.L., Kyritsis, C., Tampe, R., and Cresswell, P. (2003). Early phagosomes in dendritic cells form a cellular compartment sufficient for cross presentation of exogenous antigens. Proceedings of the National Academy of Sciences of the United States of America *100*, 12889-12894.

Acosta-Rodriguez, E.V., Rivino, L., Geginat, J., Jarrossay, D., Gattorno, M., Lanzavecchia, A., Sallusto, F., and Napolitani, G. (2007). Surface phenotype and antigenic specificity of human interleukin 17-producing T helper memory cells. Nature immunology *8*, 639-646.

Adams, J.M., and Cory, S. (1998). The Bcl-2 protein family: arbiters of cell survival. Science (New York, NY *281*, 1322-1326.

Ahn, K., Angulo, A., Ghazal, P., Peterson, P.A., Yang, Y., and Fruh, K. (1996a). Human cytomegalovirus inhibits antigen presentation by a sequential multistep process. Proceedings of the National Academy of Sciences of the United States of America *93*, 10990-10995.

Ahn, K., Gruhler, A., Galocha, B., Jones, T.R., Wiertz, E.J., Ploegh, H.L., Peterson, P.A., Yang, Y., and Fruh, K. (1997). The ER-luminal domain of the HCMV glycoprotein US6 inhibits peptide translocation by TAP. Immunity *6*, 613-621.

Ahn, K., Meyer, T.H., Uebel, S., Sempe, P., Djaballah, H., Yang, Y., Peterson, P.A., Fruh, K., and Tampe, R. (1996b). Molecular mechanism and species specificity of TAP inhibition by herpes simplex virus ICP47. The EMBO journal *15*, 3247-3255.

Akashi, K., Kondo, M., von Freeden-Jeffry, U., Murray, R., and Weissman, I.L. (1997). Bcl-2 rescues T lymphopoiesis in interleukin-7 receptor-deficient mice. Cell *89*, 1033-1041.

Alatrakchi, N., Graham, C.S., van der Vliet, H.J., Sherman, K.E., Exley, M.A., and Koziel, M.J. (2007). Hepatitis C virus (HCV)-specific CD8(+) cells produce transforming growth factor beta that can suppress HCV-specific T-cell responses. Journal of virology *81*, 5882-5892.

Albert, M.L., Sauter, B., and Bhardwaj, N. (1998). Dendritic cells acquire antigen from apoptotic cells and induce class I-restricted CTLs. Nature *392*, 86-89.

Alcami, A. (2003). Viral mimicry of cytokines, chemokines and their receptors. Nature reviews *3*, 36-50.

Allan, R.S., Smith, C.M., Belz, G.T., van Lint, A.L., Wakim, L.M., Heath, W.R., and Carbone, F.R. (2003). Epidermal viral immunity induced by CD8alpha+ dendritic cells but not by Langerhans cells. Science (New York, NY *301*, 1925-1928.

Allan, R.S., Waithman, J., Bedoui, S., Jones, C.M., Villadangos, J.A., Zhan, Y., Lew, A.M., Shortman, K., Heath, W.R., and Carbone, F.R. (2006). Migratory dendritic

cells transfer antigen to a lymph node-resident dendritic cell population for efficient CTL priming. Immunity *25*, 153-162.

Allen, L.A., and Aderem, A. (1996). Mechanisms of phagocytosis. Current opinion in immunology *8*, 36-40.

Alward, W.L., McMahon, B.J., Hall, D.B., Heyward, W.L., Francis, D.P., and Bender, T.R. (1985). The long-term serological course of asymptomatic hepatitis B virus carriers and the development of primary hepatocellular carcinoma. The Journal of infectious diseases *151*, 604-609.

Anderson, C.C., Carroll, J.M., Gallucci, S., Ridge, J.P., Cheever, A.W., and Matzinger, P. (2001). Testing time-, ignorance-, and danger-based models of tolerance. J Immunol *166*, 3663-3671.

Andonegui, G., Bonder, C.S., Green, F., Mullaly, S.C., Zbytnuik, L., Raharjo, E., and Kubes, P. (2003). Endothelium-derived Toll-like receptor-4 is the key molecule in LPS-induced neutrophil sequestration into lungs. The Journal of clinical investigation *111*, 1011-1020.

Anton, L.C., Yewdell, J.W., and Bennink, J.R. (1997). MHC class I-associated peptides produced from endogenous gene products with vastly different efficiencies. J Immunol *158*, 2535-2542.

Appay, V., Nixon, D.F., Donahoe, S.M., Gillespie, G.M., Dong, T., King, A., Ogg, G.S., Spiegel, H.M., Conlon, C., Spina, C.A., *et al.* (2000). HIV-specific CD8(+) T cells produce antiviral cytokines but are impaired in cytolytic function. The Journal of experimental medicine *192*, 63-75.

Arnold, R., Brenner, D., Becker, M., Frey, C.R., and Krammer, P.H. (2006). How T lymphocytes switch between life and death. European journal of immunology *36*, 1654-1658.

Ashkenazi, A., and Dixit, V.M. (1998). Death receptors: signaling and modulation. Science (New York, NY *281*, 1305-1308.

Ashton-Rickardt, P.G. (2005). The granule pathway of programmed cell death. Critical reviews in immunology *25*, 161-182.

Ashwell, J.D., Lu, F.W., and Vacchio, M.S. (2000). Glucocorticoids in T cell development and function*. Annual review of immunology *18*, 309-345.

Asselin-Paturel, C., Boonstra, A., Dalod, M., Durand, I., Yessaad, N., Dezutter-Dambuyant, C., Vicari, A., O'Garra, A., Biron, C., Briere, F., *et al.* (2001). Mouse type I IFN-producing cells are immature APCs with plasmacytoid morphology. Nature immunology *2*, 1144-1150.

Attuil, V., Bucher, P., Rossi, M., Mutin, M., and Maryanski, J.L. (2000). Comparative T cell receptor repertoire selection by antigen after adoptive transfer: a glimpse at an antigen-specific preimmune repertoire. Proceedings of the National Academy of Sciences of the United States of America *97*, 8473-8478.

Auffermann-Gretzinger, S., Keeffe, E.B., and Levy, S. (2001). Impaired dendritic cell maturation in patients with chronic, but not resolved, hepatitis C virus infection. Blood *97*, 3171-3176.

Bachmann, M.F., Gallimore, A., Linkert, S., Cerundolo, V., Lanzavecchia, A., Kopf, M., and Viola, A. (1999a). Developmental regulation of Lck targeting to the CD8 coreceptor controls signaling in naive and memory T cells. The Journal of experimental medicine *189*, 1521-1530.

Bachmann, M.F., Oxenius, A., Pircher, H., Hengartner, H., Ashton-Richardt, P.A., Tonegawa, S., and Zinkernagel, R.M. (1995). TAP1-independent loading of class I molecules by exogenous viral proteins. European journal of immunology *25*, 1739-1743.

Bachmann, M.F., Speiser, D.E., Mak, T.W., and Ohashi, P.S. (1999b). Absence of co-stimulation and not the intensity of TCR signaling is critical for the induction of T cell unresponsiveness in vivo. European journal of immunology *29*, 2156-2166.

Bachmann, M.F., Wolint, P., Schwarz, K., Jager, P., and Oxenius, A. (2005a). Functional properties and lineage relationship of CD8+ T cell subsets identified by expression of IL-7 receptor alpha and CD62L. J Immunol *175*, 4686-4696.

Bachmann, M.F., Wolint, P., Schwarz, K., and Oxenius, A. (2005b). Recall proliferation potential of memory CD8+ T cells and antiviral protection. J Immunol *175*, 4677-4685.

Badovinac, V.P., and Harty, J.T. (2002). CD8(+) T-cell homeostasis after infection: setting the 'curve'. Microbes and infection / Institut Pasteur *4*, 441-447.

Badovinac, V.P., Porter, B.B., and Harty, J.T. (2002). Programmed contraction of CD8(+) T cells after infection. Nature immunology *3*, 619-626.

Banchereau, J., and Steinman, R.M. (1998). Dendritic cells and the control of immunity. Nature *392*, 245-252.

Banks, T.A., Rickert, S., and Ware, C.F. (2006). Restoring immune defenses via lymphotoxin signaling: lessons from cytomegalovirus. Immunologic research *34*, 243-254.

Barber, G.N. (2001). Host defense, viruses and apoptosis. Cell death and differentiation *8*, 113-126.

Barboza, L., Salmen, S., Goncalves, L., Colmenares, M., Peterson, D., Montes, H., Cartagirone, R., Gutierrez, M.D., and Berrueta, L. (2007). Antigen-induced regulatory T cells in HBV chronically infected patients. Virology.

Barnes, E., Ward, S.M., Kasprowicz, V.O., Dusheiko, G., Klenerman, P., and Lucas, M. (2004). Ultra-sensitive class I tetramer analysis reveals previously undetectable populations of antiviral CD8+ T cells. European journal of immunology *34*, 1570-1577.

Barouch, D., Friede, T., Stevanovic, S., Tussey, L., Smith, K., Rowland-Jones, S., Braud, V., McMichael, A., and Rammensee, H.G. (1995). HLA-A2 subtypes are

functionally distinct in peptide binding and presentation. The Journal of experimental medicine *182*, 1847-1856.

Baugh, L.R., Hill, A.A., Brown, E.L., and Hunter, C.P. (2001). Quantitative analysis of mRNA amplification by in vitro transcription. Nucleic acids research *29*, E29.

Beasley, R.P., Hwang, L.Y., Lee, G.C., Lan, C.C., Roan, C.H., Huang, F.Y., and Chen, C.L. (1983). Prevention of perinatally transmitted hepatitis B virus infections with hepatitis B virus infections with hepatitis B immune globulin and hepatitis B vaccine. Lancet *2*, 1099-1102.

Behm-Ansmant, I., Rehwinkel, J., Doerks, T., Stark, A., Bork, P., and Izaurralde, E. (2006). mRNA degradation by miRNAs and GW182 requires both CCR4:NOT deadenylase and DCP1:DCP2 decapping complexes. Genes & development *20*, 1885-1898.

Behzad, H., Jamil, S., Denny, T.A., and Duronio, V. (2007). Cytokine-mediated FOXO3a phosphorylation suppresses FasL expression in hemopoietic cell lines: investigations of the role of Fas in apoptosis due to cytokine starvation. Cytokine *38*, 74-83.

Belkaid, Y., Von Stebut, E., Mendez, S., Lira, R., Caler, E., Bertholet, S., Udey, M.C., and Sacks, D. (2002). CD8+ T cells are required for primary immunity in C57BL/6 mice following low-dose, intradermal challenge with Leishmania major. J Immunol *168*, 3992-4000.

Belz, G.T., Behrens, G.M., Smith, C.M., Miller, J.F., Jones, C., Lejon, K., Fathman, C.G., Mueller, S.N., Shortman, K., Carbone, F.R., *et al.* (2002a). The CD8alpha(+) dendritic cell is responsible for inducing peripheral self-tolerance to tissue-associated antigens. The Journal of experimental medicine *196*, 1099-1104.

Belz, G.T., Carbone, F.R., and Heath, W.R. (2002b). Cross-presentation of antigens by dendritic cells. Critical reviews in immunology *22*, 439-448.

Belz, G.T., Heath, W.R., and Carbone, F.R. (2002c). The role of dendritic cell subsets in selection between tolerance and immunity. Immunol Cell Biol *80*, 463-468.

Bendelac, A., and Medzhitov, R. (2002). Adjuvants of immunity: harnessing innate immunity to promote adaptive immunity. The Journal of experimental medicine *195*, F19-23.

Benedict, C.A., Norris, P.S., and Ware, C.F. (2002). To kill or be killed: viral evasion of apoptosis. Nature immunology *3*, 1013-1018.

Bengsch, B., Spangenberg, H.C., Kersting, N., Neumann-Haefelin, C., Panther, E., von Weizsacker, F., Blum, H.E., Pircher, H., and Thimme, R. (2007). Analysis of CD127 and KLRG1 expression on hepatitis C virus-specific CD8+ T cells reveals the existence of different memory T-cell subsets in the peripheral blood and liver. Journal of virology *81*, 945-953.

Benjamini, Y., and Hochberg, Y. (1995). Controlling the false discovery rate:A practical and powerful approach to multiple testing. Journal of the Royal Statistical Society *57*, 289-300.

Bennett, S.R., Carbone, F.R., Karamalis, F., Flavell, R.A., Miller, J.F., and Heath, W.R. (1998). Help for cytotoxic-T-cell responses is mediated by CD40 signalling. Nature *393*, 478-480.

Berg, M., Wingender, G., Djandji, D., Hegenbarth, S., Momburg, F., Hammerling, G., Limmer, A., and Knolle, P. (2006). Cross-presentation of antigens from apoptotic tumor cells by liver sinusoidal endothelial cells leads to tumor-specific CD8+ T cell tolerance. European journal of immunology *36*, 2960-2970.

Bergmann, C.C., Altman, J.D., Hinton, D., and Stohlman, S.A. (1999). Inverted immunodominance and impaired cytolytic function of CD8+ T cells during viral persistence in the central nervous system. J Immunol *163*, 3379-3387.

Berke, G. (1995). The CTL's kiss of death. Cell *81*, 9-12.

Bernard, H.U. (1994). Coevolution of papillomaviruses with human populations. Trends in microbiology *2*, 140-143.

Bertoletti, A., Chisari, F.V., Penna, A., Guilhot, S., Galati, L., Missale, G., Fowler, P., Schlicht, H.J., Vitiello, A., Chesnut, R.C., *et al.* (1993). Definition of a minimal optimal cytotoxic T-cell epitope within the hepatitis B virus nucleocapsid protein. Journal of virology *67*, 2376-2380.

Bertoletti, A., Costanzo, A., Chisari, F.V., Levrero, M., Artini, M., Sette, A., Penna, A., Giuberti, T., Fiaccadori, F., and Ferrari, C. (1994). Cytotoxic T lymphocyte response to a wild type hepatitis B virus epitope in patients chronically infected by variant viruses carrying substitutions within the epitope. The Journal of experimental medicine *180*, 933-943.

Bertoletti, A., D'Elios, M.M., Boni, C., De Carli, M., Zignego, A.L., Durazzo, M., Missale, G., Penna, A., Fiaccadori, F., Del Prete, G., *et al.* (1997a). Different cytokine profiles of intraphepatic T cells in chronic hepatitis B and hepatitis C virus infections. Gastroenterology *112*, 193-199.

Bertoletti, A., Ferrari, C., Fiaccadori, F., Penna, A., Margolskee, R., Schlicht, H.J., Fowler, P., Guilhot, S., and Chisari, F.V. (1991). HLA class I-restricted human cytotoxic T cells recognize endogenously synthesized hepatitis B virus nucleocapsid antigen. Proceedings of the National Academy of Sciences of the United States of America *88*, 10445-10449.

Bertoletti, A., Southwood, S., Chesnut, R., Sette, A., Falco, M., Ferrara, G.B., Penna, A., Boni, C., Fiaccadori, F., and Ferrari, C. (1997b). Molecular features of the hepatitis B virus nucleocapsid T-cell epitope 18-27: interaction with HLA and T-cell receptor. Hepatology (Baltimore, Md *26*, 1027-1034.

Bertolino, P., Bowen, D.G., McCaughan, G.W., and Fazekas de St Groth, B. (2001). Antigen-specific primary activation of CD8+ T cells within the liver. J Immunol *166*, 5430-5438.

Bertolino, P., Trescol-Biemont, M.C., and Rabourdin-Combe, C. (1998). Hepatocytes induce functional activation of naive CD8+ T lymphocytes but fail to promote survival. European journal of immunology *28*, 221-236.

Bertoni, R., Sidney, J., Fowler, P., Chesnut, R.W., Chisari, F.V., and Sette, A. (1997). Human histocompatibility leukocyte antigen-binding supermotifs predict broadly cross-reactive cytotoxic T lymphocyte responses in patients with acute hepatitis. The Journal of clinical investigation *100*, 503-513.

Bertram, E.M., Dawicki, W., Sedgmen, B., Bramson, J.L., Lynch, D.H., and Watts, T.H. (2004a). A switch in costimulation from CD28 to 4-1BB during primary versus secondary CD8 T cell response to influenza in vivo. J Immunol *172*, 981-988.

Bertram, E.M., Dawicki, W., and Watts, T.H. (2004b). Role of T cell costimulation in anti-viral immunity. Seminars in immunology *16*, 185-196.

Bevan, M.J. (1987). Antigen recognition. Class discrimination in the world of immunology. Nature *325*, 192-194.

Bieganowska, K., Hollsberg, P., Buckle, G.J., Lim, D.G., Greten, T.F., Schneck, J., Altman, J.D., Jacobson, S., Ledis, S.L., Hanchard, B., *et al.* (1999). Direct analysis of viral-specific CD8+ T cells with soluble HLA-A2/Tax11-19 tetramer complexes in patients with human T cell lymphotropic virus-associated myelopathy. J Immunol *162*, 1765-1771.

Biermer, M., Puro, R., and Schneider, R.J. (2003). Tumor necrosis factor alpha inhibition of hepatitis B virus replication involves disruption of capsid Integrity through activation of NF-kappaB. Journal of virology *77*, 4033-4042.

Bjorkman, P.J., Saper, M.A., Samraoui, B., Bennett, W.S., Strominger, J.L., and Wiley, D.C. (1987a). Structure of the human class I histocompatibility antigen, HLA-A2. Nature *329*, 506-512.

Bjorkman, P.J., Saper, M.A., Samraoui, B., Bennett, W.S., Strominger, J.L., and Wiley, D.C. (1987b). The foreign antigen binding site and T cell recognition regions of class I histocompatibility antigens. Nature *329*, 512-518.

Blohm, U., Roth, E., Brommer, K., Dumrese, T., Rosenthal, F.M., and Pircher, H. (2002). Lack of effector cell function and altered tetramer binding of tumor-infiltrating lymphocytes. J Immunol *169*, 5522-5530.

Bocher, W.O., Herzog-Hauff, S., Schlaak, J., Meyer zum Buschenfeld, K.H., and Lohr, H.F. (1999). Kinetics of hepatitis B surface antigen-specific immune responses in acute and chronic hepatitis B or after HBs vaccination: stimulation of the in vitro antibody response by interferon gamma. Hepatology (Baltimore, Md *29*, 238-244.

Boise, L.H., Gonzalez-Garcia, M., Postema, C.E., Ding, L., Lindsten, T., Turka, L.A., Mao, X., Nunez, G., and Thompson, C.B. (1993). bcl-x, a bcl-2-related gene that functions as a dominant regulator of apoptotic cell death. Cell *74*, 597-608.

Boldin, M.P., Goncharov, T.M., Goltsev, Y.V., and Wallach, D. (1996). Involvement of MACH, a novel MORT1/FADD-interacting protease, in Fas/APO-1- and TNF receptor-induced cell death. Cell *85*, 803-815.

Boldin, M.P., Mett, I.L., Varfolomeev, E.E., Chumakov, I., Shemer-Avni, Y., Camonis, J.H., and Wallach, D. (1995a). Self-association of the "death domains" of the p55 tumor necrosis factor (TNF) receptor and Fas/APO1 prompts signaling for TNF and Fas/APO1 effects. The Journal of biological chemistry *270*, 387-391.

Boldin, M.P., Varfolomeev, E.E., Pancer, Z., Mett, I.L., Camonis, J.H., and Wallach, D. (1995b). A novel protein that interacts with the death domain of Fas/APO1 contains a sequence motif related to the death domain. The Journal of biological chemistry *270*, 7795-7798.

Bonasio, R., Scimone, M.L., Schaerli, P., Grabie, N., Lichtman, A.H., and von Andrian, U.H. (2006). Clonal deletion of thymocytes by circulating dendritic cells homing to the thymus. Nature immunology *7*, 1092-1100.

Bond, W.W., Favero, M.S., Petersen, N.J., Gravelle, C.R., Ebert, J.W., and Maynard, J.E. (1981). Survival of hepatitis B virus after drying and storage for one week. Lancet *1*, 550-551.

Boni, C., Fisicaro, P., Valdatta, C., Amadei, B., Di Vincenzo, P., Giuberti, T., Laccabue, D., Zerbini, A., Cavalli, A., Missale, G., *et al.* (2007). Characterization of hepatitis B virus (HBV)-specific T-cell dysfunction in chronic HBV infection. Journal of virology *81*, 4215-4225.

Boni, C., Penna, A., Bertoletti, A., Lamonaca, V., Rapti, I., Missale, G., Pilli, M., Urbani, S., Cavalli, A., Cerioni, S., *et al.* (2003). Transient restoration of anti-viral T cell responses induced by lamivudine therapy in chronic hepatitis B. Journal of hepatology *39*, 595-605.

Bouillet, P., Metcalf, D., Huang, D.C., Tarlinton, D.M., Kay, T.W., Kontgen, F., Adams, J.M., and Strasser, A. (1999). Proapoptotic Bcl-2 relative Bim required for certain apoptotic responses, leukocyte homeostasis, and to preclude autoimmunity. Science (New York, NY *286*, 1735-1738.

Bouillet, P., Purton, J.F., Godfrey, D.I., Zhang, L.C., Coultas, L., Puthalakath, H., Pellegrini, M., Cory, S., Adams, J.M., and Strasser, A. (2002). BH3-only Bcl-2 family member Bim is required for apoptosis of autoreactive thymocytes. Nature *415*, 922-926.

Boussiotis, V.A., Barber, D.L., Lee, B.J., Gribben, J.G., Freeman, G.J., and Nadler, L.M. (1996). Differential association of protein tyrosine kinases with the T cell receptor is linked to the induction of anergy and its prevention by B7 family-mediated costimulation. The Journal of experimental medicine *184*, 365-376.

Boussiotis, V.A., Freeman, G.J., Taylor, P.A., Berezovskaya, A., Grass, I., Blazar, B.R., and Nadler, L.M. (2000a). p27kip1 functions as an anergy factor inhibiting interleukin 2 transcription and clonal expansion of alloreactive human and mouse helper T lymphocytes. Nature medicine *6*, 290-297.

Boussiotis, V.A., Tsai, E.Y., Yunis, E.J., Thim, S., Delgado, J.C., Dascher, C.C., Berezovskaya, A., Rousset, D., Reynes, J.M., and Goldfeld, A.E. (2000b). IL-10-producing T cells suppress immune responses in anergic tuberculosis patients. The Journal of clinical investigation *105*, 1317-1325.

Boyd, J.M., Gallo, G.J., Elangovan, B., Houghton, A.B., Malstrom, S., Avery, B.J., Ebb, R.G., Subramanian, T., Chittenden, T., Lutz, R.J., *et al.* (1995). Bik, a novel death-inducing protein shares a distinct sequence motif with Bcl-2 family proteins and interacts with viral and cellular survival-promoting proteins. Oncogene *11*, 1921-1928.

Brehm, M.A., Pinto, A.K., Daniels, K.A., Schneck, J.P., Welsh, R.M., and Selin, L.K. (2002). T cell immunodominance and maintenance of memory regulated by unexpectedly cross-reactive pathogens. Nature immunology *3*, 627-634.

Breiman, A., Grandvaux, N., Lin, R., Ottone, C., Akira, S., Yoneyama, M., Fujita, T., Hiscott, J., and Meurs, E.F. (2005). Inhibition of RIG-I-dependent signaling to the interferon pathway during hepatitis C virus expression and restoration of signaling by IKKepsilon. Journal of virology *79*, 3969-3978.

Brierley, M.M., and Fish, E.N. (2002). Review: IFN-alpha/beta receptor interactions to biologic outcomes: understanding the circuitry. J Interferon Cytokine Res *22*, 835-845.

Brooks, D.G., McGavern, D.B., and Oldstone, M.B. (2006a). Reprogramming of antiviral T cells prevents inactivation and restores T cell activity during persistent viral infection. The Journal of clinical investigation *116*, 1675-1685.

Brooks, D.G., Trifilo, M.J., Edelmann, K.H., Teyton, L., McGavern, D.B., and Oldstone, M.B. (2006b). Interleukin-10 determines viral clearance or persistence in vivo. Nature medicine *12*, 1301-1309.

Browning, M., and Krausa, P. (1996). Genetic diversity of HLA-A2: evolutionary and functional significance. Immunology today *17*, 165-170.

Burk, R.D., Hwang, L.Y., Ho, G.Y., Shafritz, D.A., and Beasley, R.P. (1994). Outcome of perinatal hepatitis B virus exposure is dependent on maternal virus load. The Journal of infectious diseases *170*, 1418-1423.

Busch, D.H., and Pamer, E.G. (1998). MHC class I/peptide stability: implications for immunodominance, in vitro proliferation, and diversity of responding CTL. J Immunol *160*, 4441-4448.

Butz, E.A., and Bevan, M.J. (1998). Massive expansion of antigen-specific CD8+ T cells during an acute virus infection. Immunity *8*, 167-175.

Cantin, R., Methot, S., and Tremblay, M.J. (2005). Plunder and stowaways: incorporation of cellular proteins by enveloped viruses. Journal of virology *79*, 6577-6587.

Carbone, F.R., Belz, G.T., and Heath, W.R. (2004). Transfer of antigen between migrating and lymph node-resident DCs in peripheral T-cell tolerance and immunity. Trends in immunology *25*, 655-658.

Carbone, F.R., and Bevan, M.J. (1990). Class I-restricted processing and presentation of exogenous cell-associated antigen in vivo. The Journal of experimental medicine *171*, 377-387.

Carman, W.F., Zanetti, A.R., Karayiannis, P., Waters, J., Manzillo, G., Tanzi, E., Zuckerman, A.J., and Thomas, H.C. (1990). Vaccine-induced escape mutant of hepatitis B virus. Lancet *336*, 325-329.

Carrier, Y., Yuan, J., Kuchroo, V.K., and Weiner, H.L. (2007a). Th3 cells in peripheral tolerance. I. Induction of Foxp3-positive regulatory T cells by Th3 cells derived from TGF-beta T cell-transgenic mice. J Immunol *178*, 179-185.

Carrier, Y., Yuan, J., Kuchroo, V.K., and Weiner, H.L. (2007b). Th3 cells in peripheral tolerance. II. TGF-beta-transgenic Th3 cells rescue IL-2-deficient mice from autoimmunity. J Immunol *178*, 172-178.

Catalfamo, M., and Henkart, P.A. (2003). Perforin and the granule exocytosis cytotoxicity pathway. Current opinion in immunology *15*, 522-527.

Celis, E., Ou, D., and Otvos, L., Jr. (1988). Recognition of hepatitis B surface antigen by human T lymphocytes. Proliferative and cytotoxic responses to a major antigenic determinant defined by synthetic peptides. J Immunol *140*, 1808-1815.

Cella, M., Jarrossay, D., Facchetti, F., Alebardi, O., Nakajima, H., Lanzavecchia, A., and Colonna, M. (1999). Plasmacytoid monocytes migrate to inflamed lymph nodes and produce large amounts of type I interferon. Nature medicine *5*, 919-923.

Cerwenka, A., and Lanier, L.L. (2001). Natural killer cells, viruses and cancer. Nature reviews *1*, 41-49.

Chan, A.C., Iwashima, M., Turck, C.W., and Weiss, A. (1992). ZAP-70: a 70 kd protein-tyrosine kinase that associates with the TCR zeta chain. Cell *71*, 649-662.

Chang, J.J., Wightman, F., Bartholomeusz, A., Ayres, A., Kent, S.J., Sasadeusz, J., and Lewin, S.R. (2005). Reduced hepatitis B virus (HBV)-specific CD4+ T-cell responses in human immunodeficiency virus type 1-HBV-coinfected individuals receiving HBV-active antiretroviral therapy. Journal of virology *79*, 3038-3051.

Chang, M.H. (2000). Natural history of hepatitis B virus infection in children. Journal of gastroenterology and hepatology *15 Suppl*, E16-19.

Chen, D., and Zhou, Q. (2004). Caspase cleavage of BimEL triggers a positive feedback amplification of apoptotic signaling. Proceedings of the National Academy of Sciences of the United States of America *101*, 1235-1240.

Chen, H.D., Fraire, A.E., Joris, I., Brehm, M.A., Welsh, R.M., and Selin, L.K. (2001). Memory CD8+ T cells in heterologous antiviral immunity and immunopathology in the lung. Nature immunology *2*, 1067-1076.

Chen, H.S., Kew, M.C., Hornbuckle, W.E., Tennant, B.C., Cote, P.J., Gerin, J.L., Purcell, R.H., and Miller, R.H. (1992). The precore gene of the woodchuck hepatitis

virus genome is not essential for viral replication in the natural host. Journal of virology *66*, 5682-5684.

Chen, M., Sallberg, M., Hughes, J., Jones, J., Guidotti, L.G., Chisari, F.V., Billaud, J.N., and Milich, D.R. (2005). Immune tolerance split between hepatitis B virus precore and core proteins. Journal of virology *79*, 3016-3027.

Chen, W., Anton, L.C., Bennink, J.R., and Yewdell, J.W. (2000). Dissecting the multifactorial causes of immunodominance in class I-restricted T cell responses to viruses. Immunity *12*, 83-93.

Cheng, E.H., Wei, M.C., Weiler, S., Flavell, R.A., Mak, T.W., Lindsten, T., and Korsmeyer, S.J. (2001). BCL-2, BCL-X(L) sequester BH3 domain-only molecules preventing BAX- and BAK-mediated mitochondrial apoptosis. Molecular cell *8*, 705-711.

Chien, Y.H., and Konigshofer, Y. (2007). Antigen recognition by gammadelta T cells. Immunological reviews *215*, 46-58.

Chinnaiyan, A.M., O'Rourke, K., Tewari, M., and Dixit, V.M. (1995). FADD, a novel death domain-containing protein, interacts with the death domain of Fas and initiates apoptosis. Cell *81*, 505-512.

Chisari, F.V., and Ferrari, C. (1995). Hepatitis B virus immunopathogenesis. Annual review of immunology *13*, 29-60.

Chittenden, T., Flemington, C., Houghton, A.B., Ebb, R.G., Gallo, G.J., Elangovan, B., Chinnadurai, G., and Lutz, R.J. (1995). A conserved domain in Bak, distinct from BH1 and BH2, mediates cell death and protein binding functions. The EMBO journal *14*, 5589-5596.

Chomczynski, P., and Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Analytical biochemistry *162*, 156-159.

Chtanova, T., Tangye, S.G., Newton, R., Frank, N., Hodge, M.R., Rolph, M.S., and Mackay, C.R. (2004). T follicular helper cells express a distinctive transcriptional profile, reflecting their role as non-Th1/Th2 effector cells that provide help for B cells. J Immunol *173*, 68-78.

Cohen, G.B., Gandhi, R.T., Davis, D.M., Mandelboim, O., Chen, B.K., Strominger, J.L., and Baltimore, D. (1999). The selective downregulation of class I major histocompatibility complex proteins by HIV-1 protects HIV-infected cells from NK cells. Immunity *10*, 661-671.

Colamonici, O.R., Domanski, P., Sweitzer, S.M., Larner, A., and Buller, R.M. (1995). Vaccinia virus B18R gene encodes a type I interferon-binding protein that blocks interferon alpha transmembrane signaling. The Journal of biological chemistry *270*, 15974-15978.

Cooper, C.L., Davis, H.L., Angel, J.B., Morris, M.L., Elfer, S.M., Seguin, I., Krieg, A.M., and Cameron, D.W. (2005). CPG 7909 adjuvant improves hepatitis B virus vaccine seroprotection in antiretroviral-treated HIV-infected adults. AIDS (London, England) *19*, 1473-1479.

Cooper, C.L., Davis, H.L., Morris, M.L., Efler, S.M., Adhami, M.A., Krieg, A.M., Cameron, D.W., and Heathcote, J. (2004). CPG 7909, an immunostimulatory TLR9 agonist oligodeoxynucleotide, as adjuvant to Engerix-B HBV vaccine in healthy adults: a double-blind phase I/II study. Journal of clinical immunology *24*, 693-701.

Cornberg, M., Chen, A.T., Wilkinson, L.A., Brehm, M.A., Kim, S.K., Calcagno, C., Ghersi, D., Puzone, R., Celada, F., Welsh, R.M., *et al.* (2006). Narrowed TCR repertoire and viral escape as a consequence of heterologous immunity. The Journal of clinical investigation *116*, 1443-1456.

Cornberg, M., Sheridan, B.S., Saccoccio, F.M., Brehm, M.A., and Selin, L.K. (2007). Protection against vaccinia virus challenge by CD8 memory T cells resolved by molecular mimicry. Journal of virology *81*, 934-944.

Cresswell, P., Bangia, N., Dick, T., and Diedrich, G. (1999). The nature of the MHC class I peptide loading complex. Immunological reviews *172*, 21-28.

Croft, M. (2003). Co-stimulatory members of the TNFR family: keys to effective T-cell immunity? Nature reviews *3*, 609-620.

Crotzer, V.L., Christian, R.E., Brooks, J.M., Shabanowitz, J., Settlage, R.E., Marto, J.A., White, F.M., Rickinson, A.B., Hunt, D.F., and Engelhard, V.H. (2000). Immunodominance among EBV-derived epitopes restricted by HLA-B27 does not correlate with epitope abundance in EBV-transformed B-lymphoblastoid cell lines. J Immunol *164*, 6120-6129.

Davey, G.M., Kurts, C., Miller, J.F., Bouillet, P., Strasser, A., Brooks, A.G., Carbone, F.R., and Heath, W.R. (2002). Peripheral deletion of autoreactive CD8 T cells by cross presentation of self-antigen occurs by a Bcl-2-inhibitable pathway mediated by Bim. The Journal of experimental medicine *196*, 947-955.

de Franchis, R., Meucci, G., Vecchi, M., Tatarella, M., Colombo, M., Del Ninno, E., Rumi, M.G., Donato, M.F., and Ronchi, G. (1993). The natural history of asymptomatic hepatitis B surface antigen carriers. Annals of internal medicine *118*, 191-194.

Debrick, J.E., Campbell, P.A., and Staerz, U.D. (1991). Macrophages as accessory cells for class I MHC-restricted immune responses. J Immunol *147*, 2846-2851.

Demotte, N., Colau, D., Ottaviani, S., Godelaine, D., Van Pel, A., Boon, T., and van der Bruggen, P. (2002). A reversible functional defect of CD8+ T lymphocytes involving loss of tetramer labeling. European journal of immunology *32*, 1688-1697.

den Haan, J.M., and Bevan, M.J. (2002). Constitutive versus activation-dependent cross-presentation of immune complexes by CD8(+) and CD8(-) dendritic cells in vivo. The Journal of experimental medicine *196*, 817-827.

den Haan, J.M., Kraal, G., and Bevan, M.J. (2007). Cutting edge: Lipopolysaccharide induces IL-10-producing regulatory CD4+ T cells that suppress the CD8+ T cell response. J Immunol *178*, 5429-5433.

den Haan, J.M., Lehar, S.M., and Bevan, M.J. (2000). CD8(+) but not CD8(-) dendritic cells cross-prime cytotoxic T cells in vivo. The Journal of experimental medicine *192*, 1685-1696.

Derbinski, J., Schulte, A., Kyewski, B., and Klein, L. (2001). Promiscuous gene expression in medullary thymic epithelial cells mirrors the peripheral self. Nature immunology *2*, 1032-1039.

DeSilva, D.R., Urdahl, K.B., and Jenkins, M.K. (1991). Clonal anergy is induced in vitro by T cell receptor occupancy in the absence of proliferation. J Immunol *147*, 3261-3267.

Dheda, K., Huggett, J.F., Bustin, S.A., Johnson, M.A., Rook, G., and Zumla, A. (2004). Validation of housekeeping genes for normalizing RNA expression in real-time PCR. BioTechniques *37*, 112-114, 116, 118-119.

Dheda, K., Huggett, J.F., Chang, J.S., Kim, L.U., Bustin, S.A., Johnson, M.A., Rook, G.A., and Zumla, A. (2005). The implications of using an inappropriate reference gene for real-time reverse transcription PCR data normalization. Analytical biochemistry *344*, 141-143.

Diebold, S.S., Kaisho, T., Hemmi, H., Akira, S., and Reis e Sousa, C. (2004). Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. Science (New York, NY *303*, 1529-1531.

Diebold, S.S., Montoya, M., Unger, H., Alexopoulou, L., Roy, P., Haswell, L.E., Al-Shamkhani, A., Flavell, R., Borrow, P., and Reis e Sousa, C. (2003). Viral infection switches non-plasmacytoid dendritic cells into high interferon producers. Nature *424*, 324-328.

Dijkers, P.F., Medema, R.H., Lammers, J.W., Koenderman, L., and Coffer, P.J. (2000). Expression of the pro-apoptotic Bcl-2 family member Bim is regulated by the forkhead transcription factor FKHR-L1. Curr Biol *10*, 1201-1204.

Donaghy, H., Gazzard, B., Gotch, F., and Patterson, S. (2003). Dysfunction and infection of freshly isolated blood myeloid and plasmacytoid dendritic cells in patients infected with HIV-1. Blood *101*, 4505-4511.

Duggan, D.J., Bittner, M., Chen, Y., Meltzer, P., and Trent, J.M. (1999). Expression profiling using cDNA microarrays. Nature genetics *21*, 10-14.

Dunn, C., Brunetto, M., Reynolds, G., Christophides, T., Kennedy, P.T., Lampertico, P., Das, A., Lopes, A.R., Borrow, P., Williams, K., *et al.* (2007). Cytokines induced during chronic hepatitis B virus infection promote a pathway for NK cell-mediated liver damage. The Journal of experimental medicine *204*, 667-680.

Eckmann, L., Stenson, W.F., Savidge, T.C., Lowe, D.C., Barrett, K.E., Fierer, J., Smith, J.R., and Kagnoff, M.F. (1997). Role of intestinal epithelial cells in the host secretory response to infection by invasive bacteria. Bacterial entry induces epithelial prostaglandin h synthase-2 expression and prostaglandin E2 and F2alpha production. The Journal of clinical investigation *100*, 296-309.

Eisen, M.B., Spellman, P.T., Brown, P.O., and Botstein, D. (1998). Cluster analysis and display of genome-wide expression patterns. Proceedings of the National Academy of Sciences of the United States of America *95*, 14863-14868.

Ejrnaes, M., Filippi, C.M., Martinic, M.M., Ling, E.M., Togher, L.M., Crotty, S., and von Herrath, M.G. (2006). Resolution of a chronic viral infection after interleukin-10 receptor blockade. The Journal of experimental medicine *203*, 2461-2472.

Elrefaei, M., Ventura, F.L., Baker, C.A., Clark, R., Bangsberg, D.R., and Cao, H. (2007). HIV-specific IL-10-positive CD8+ T cells suppress cytolysis and IL-2 production by CD8+ T cells. J Immunol *178*, 3265-3271.

Enari, M., Sakahira, H., Yokoyama, H., Okawa, K., Iwamatsu, A., and Nagata, S. (1998). A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD. Nature *391*, 43-50.

Falk, K., Rotzschke, O., and Rammensee, H.G. (1992). Specificity of antigen processing for MHC class I restricted presentation is conserved between mouse and man. European journal of immunology *22*, 1323-1326.

Falk, K., Rotzschke, O., Stevanovic, S., Jung, G., and Rammensee, H.G. (1991). Allele-specific motifs revealed by sequencing of self-peptides eluted from MHC molecules. Nature *351*, 290-296.

Falo, L.D., Jr., Kovacsovics-Bankowski, M., Thompson, K., and Rock, K.L. (1995). Targeting antigen into the phagocytic pathway in vivo induces protective tumour immunity. Nature medicine *1*, 649-653.

Farrell, H.E., and Davis-Poynter, N.J. (1998). From sabotage to camouflage: viral evasion of cytotoxic T lymphocyte and natural killer cell-mediated immunity. Seminars in cell & developmental biology *9*, 369-378.

Fattovich, G. (2003). Natural history and prognosis of hepatitis B. Seminars in liver disease *23*, 47-58.

Fattovich, G., Giustina, G., Sanchez-Tapias, J., Quero, C., Mas, A., Olivotto, P.G., Solinas, A., Almasio, P., Hadziyannis, S., Degos, F., *et al.* (1998). Delayed clearance of serum HBsAg in compensated cirrhosis B: relation to interferon alpha therapy and disease prognosis. European Concerted Action on Viral Hepatitis (EUROHEP). The American journal of gastroenterology *93*, 896-900.

Ferrari, C., Penna, A., Bertoletti, A., Cavalli, A., Valli, A., Missale, G., Pilli, M., Marchelli, S., Giuberti, T., and Fiaccadori, F. (1992). Immune pathogenesis of hepatitis B. Arch Virol Suppl *4*, 11-18.

Ferrari, C., Penna, A., Bertoletti, A., Cavalli, A., Valli, A., Schianchi, C., and Fiaccadori, F. (1989). The preS1 antigen of hepatitis B virus is highly immunogenic at the T cell level in man. The Journal of clinical investigation *84*, 1314-1319.

Ferrari, C., Penna, A., Bertoletti, A., Valli, A., Antoni, A.D., Giuberti, T., Cavalli, A., Petit, M.A., and Fiaccadori, F. (1990). Cellular immune response to hepatitis B virus-encoded antigens in acute and chronic hepatitis B virus infection. J Immunol *145*, 3442-3449.

Finlay, B.B., and McFadden, G. (2006). Anti-immunology: evasion of the host immune system by bacterial and viral pathogens. Cell *124*, 767-782.

Frahm, N., Kiepiela, P., Adams, S., Linde, C.H., Hewitt, H.S., Sango, K., Feeney, M.E., Addo, M.M., Lichterfeld, M., Lahaie, M.P., *et al.* (2006). Control of human immunodeficiency virus replication by cytotoxic T lymphocytes targeting subdominant epitopes. Nature immunology *7*, 173-178.

Franzese, O., Kennedy, P.T., Gehring, A.J., Gotto, J., Williams, R., Maini, M.K., and Bertoletti, A. (2005). Modulation of the CD8+-T-cell response by CD4+ CD25+ regulatory T cells in patients with hepatitis B virus infection. Journal of virology *79*, 3322-3328.

Fruh, K., Ahn, K., Djaballah, H., Sempe, P., van Endert, P.M., Tampe, R., Peterson, P.A., and Yang, Y. (1995). A viral inhibitor of peptide transporters for antigen presentation. Nature *375*, 415-418.

Fu, J., Xu, D., Liu, Z., Shi, M., Zhao, P., Fu, B., Zhang, Z., Yang, H., Zhang, H., Zhou, C., *et al.* (2007). Increased regulatory T cells correlate with CD8 T-cell impairment and poor survival in hepatocellular carcinoma patients. Gastroenterology *132*, 2328-2339.

Fuchs, E. (1992). Two signal model of lymphocyte activation. Immunology today *13*, 462.

Fujimoto, C., Nakagawa, Y., Ohara, K., and Takahashi, H. (2004). Polyriboinosinic polyribocytidylic acid [poly(I:C)]/TLR3 signaling allows class I processing of exogenous protein and induction of HIV-specific CD8+ cytotoxic T lymphocytes. International immunology *16*, 55-63.

Fuller, M.J., Hildeman, D.A., Sabbaj, S., Gaddis, D.E., Tebo, A.E., Shang, L., Goepfert, P.A., and Zajac, A.J. (2005). Cutting edge: emergence of CD127high functionally competent memory T cells is compromised by high viral loads and inadequate T cell help. J Immunol *174*, 5926-5930.

Fuller, M.J., Khanolkar, A., Tebo, A.E., and Zajac, A.J. (2004). Maintenance, loss, and resurgence of T cell responses during acute, protracted, and chronic viral infections. J Immunol *172*, 4204-4214.

Fuller, M.J., and Zajac, A.J. (2003). Ablation of CD8 and CD4 T cell responses by high viral loads. J Immunol *170*, 477-486.

Gajewski, T.F., Fields, P., and Fitch, F.W. (1995). Induction of the increased Fyn kinase activity in anergic T helper type 1 clones requires calcium and protein synthesis and is sensitive to cyclosporin A. European journal of immunology *25*, 1836-1842.

Gajewski, T.F., Qian, D., Fields, P., and Fitch, F.W. (1994). Anergic T-lymphocyte clones have altered inositol phosphate, calcium, and tyrosine kinase signaling pathways. Proceedings of the National Academy of Sciences of the United States of America *91*, 38-42.

Gallimore, A., Dumrese, T., Hengartner, H., Zinkernagel, R.M., and Rammensee, H.G. (1998a). Protective immunity does not correlate with the hierarchy of virus-specific cytotoxic T cell responses to naturally processed peptides. The Journal of experimental medicine *187*, 1647-1657.

Gallimore, A., Hengartner, H., and Zinkernagel, R. (1998b). Hierarchies of antigen-specific cytotoxic T-cell responses. Immunological reviews *164*, 29-36.

Gallimore, A., Hombach, J., Dumrese, T., Rammensee, H.G., Zinkernagel, R.M., and Hengartner, H. (1998c). A protective cytotoxic T cell response to a subdominant epitope is influenced by the stability of the MHC class I/peptide complex and the overall spectrum of viral peptides generated within infected cells. European journal of immunology *28*, 3301-3311.

Gallucci, S., and Matzinger, P. (2001). Danger signals: SOS to the immune system. Current opinion in immunology *13*, 114-119.

Galy, A., Travis, M., Cen, D., and Chen, B. (1995). Human T, B, natural killer, and dendritic cells arise from a common bone marrow progenitor cell subset. Immunity *3*, 459-473.

Garba, M.L., Pilcher, C.D., Bingham, A.L., Eron, J., and Frelinger, J.A. (2002). HIV antigens can induce TGF-beta(1)-producing immunoregulatory CD8+ T cells. J Immunol *168*, 2247-2254.

Garboczi, D.N., Ghosh, P., Utz, U., Fan, Q.R., Biddison, W.E., and Wiley, D.C. (1996). Structure of the complex between human T-cell receptor, viral peptide and HLA-A2. Nature *384*, 134-141.

Gehring, A.J., Sun, D., Kennedy, P.T., Nolte-'t Hoen, E., Lim, S.G., Wasser, S., Selden, C., Maini, M.K., Davis, D.M., Nassal, M., *et al.* (2007). The level of viral antigen presented by hepatocytes influences CD8 T-cell function. Journal of virology *81*, 2940-2949.

Geleziunas, R., Xu, W., Takeda, K., Ichijo, H., and Greene, W.C. (2001). HIV-1 Nef inhibits ASK1-dependent death signalling providing a potential mechanism for protecting the infected host cell. Nature *410*, 834-838.

Gibson, L., Holmgreen, S.P., Huang, D.C., Bernard, O., Copeland, N.G., Jenkins, N.A., Sutherland, G.R., Baker, E., Adams, J.M., and Cory, S. (1996). bcl-w, a novel member of the bcl-2 family, promotes cell survival. Oncogene *13*, 665-675.

Girard, J.P., and Springer, T.A. (1995). High endothelial venules (HEVs): specialized endothelium for lymphocyte migration. Immunology today *16*, 449-457.

Goldstein, D.R., Thomas, J.M., Kirklin, J.K., and George, J.F. (2001). Indefinite allograft survival mediated by donor bone marrow is dependent on the presence of a functional CD95 (Fas) gene in recipients. J Heart Lung Transplant *20*, 1132-1135.

Gotto, J., Webster, G.J., Brown, D., Jenkins, J., Dusheiko, G.M., and Bertoletti, A. (2006). The impact of HBV-DNA fluctuations on virus-specific CD8+ T cells in HBeAg+ chronic hepatitis B patients treated with a steroid and lamivudine. Journal of viral hepatitis *13*, 415-425.

Gougeon, M.L. (2005). To kill or be killed: how HIV exhausts the immune system. Cell death and differentiation *12 Suppl 1*, 845-854.

Gould, E.A., de Lamballerie, X., Zanotto, P.M., and Holmes, E.C. (2001). Evolution, epidemiology, and dispersal of flaviviruses revealed by molecular phylogenies. Advances in virus research *57*, 71-103.

Goulder, P.J., Altfeld, M.A., Rosenberg, E.S., Nguyen, T., Tang, Y., Eldridge, R.L., Addo, M.M., He, S., Mukherjee, J.S., Phillips, M.N., *et al.* (2001). Substantial differences in specificity of HIV-specific cytotoxic T cells in acute and chronic HIV infection. The Journal of experimental medicine *193*, 181-194.

Grayson, J.M., Weant, A.E., Holbrook, B.C., and Hildeman, D. (2006). Role of Bim in regulating CD8+ T-cell responses during chronic viral infection. Journal of virology *80*, 8627-8638.

Greenwald, R.J., Freeman, G.J., and Sharpe, A.H. (2005). The B7 family revisited. Annual review of immunology *23*, 515-548.

Greten, T.F., Slansky, J.E., Kubota, R., Soldan, S.S., Jaffee, E.M., Leist, T.P., Pardoll, D.M., Jacobson, S., and Schneck, J.P. (1998). Direct visualization of antigen-specific T cells: HTLV-1 Tax11-19- specific CD8(+) T cells are activated in peripheral blood and accumulate in cerebrospinal fluid from HAM/TSP patients.

Proceedings of the National Academy of Sciences of the United States of America *95*, 7568-7573.

Grouard, G., Rissoan, M.C., Filgueira, L., Durand, I., Banchereau, J., and Liu, Y.J. (1997). The enigmatic plasmacytoid T cells develop into dendritic cells with interleukin (IL)-3 and CD40-ligand. The Journal of experimental medicine *185*, 1101-1111.

Guermonprez, P., Saveanu, L., Kleijmeer, M., Davoust, J., Van Endert, P., and Amigorena, S. (2003). ER-phagosome fusion defines an MHC class I cross-presentation compartment in dendritic cells. Nature *425*, 397-402.

Guidotti, L.G., Ishikawa, T., Hobbs, M.V., Matzke, B., Schreiber, R., and Chisari, F.V. (1996). Intracellular inactivation of the hepatitis B virus by cytotoxic T lymphocytes. Immunity *4*, 25-36.

Guidotti, L.G., Morris, A., Mendez, H., Koch, R., Silverman, R.H., Williams, B.R., and Chisari, F.V. (2002). Interferon-regulated pathways that control hepatitis B virus replication in transgenic mice. Journal of virology *76*, 2617-2621.

Guidotti, L.G., Rochford, R., Chung, J., Shapiro, M., Purcell, R., and Chisari, F.V. (1999). Viral clearance without destruction of infected cells during acute HBV infection. Science (New York, NY *284*, 825-829.

Guo, B., Godzik, A., and Reed, J.C. (2001). Bcl-G, a novel pro-apoptotic member of the Bcl-2 family. The Journal of biological chemistry *276*, 2780-2785.

Hanahan, D. (1998). Peripheral-antigen-expressing cells in thymic medulla: factors in self-tolerance and autoimmunity. Current opinion in immunology *10*, 656-662.

Harada, H., Quearry, B., Ruiz-Vela, A., and Korsmeyer, S.J. (2004). Survival factor-induced extracellular signal-regulated kinase phosphorylates BIM, inhibiting its association with BAX and proapoptotic activity. Proceedings of the National Academy of Sciences of the United States of America *101*, 15313-15317.

Harari, A., Zimmerli, S.C., and Pantaleo, G. (2004). Cytomegalovirus (CMV)-specific cellular immune responses. Human immunology *65*, 500-506.

Harding, F.A., McArthur, J.G., Gross, J.A., Raulet, D.H., and Allison, J.P. (1992). CD28-mediated signalling co-stimulates murine T cells and prevents induction of anergy in T-cell clones. Nature *356*, 607-609.

Haring, J.S., Badovinac, V.P., and Harty, J.T. (2006). Inflaming the CD8+ T cell response. Immunity *25*, 19-29.

Harrington, L.E., Hatton, R.D., Mangan, P.R., Turner, H., Murphy, T.L., Murphy, K.M., and Weaver, C.T. (2005). Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. Nature immunology *6*, 1123-1132.

Harris, R.S., Bishop, K.N., Sheehy, A.M., Craig, H.M., Petersen-Mahrt, S.K., Watt, I.N., Neuberger, M.S., and Malim, M.H. (2003). DNA deamination mediates innate immunity to retroviral infection. Cell *113*, 803-809.

Hartigan, J.A., and Wong, M.A. (1979). A K-means clustering algorithm. Applied Statistics *28*, 100-108.

Hawiger, D., Inaba, K., Dorsett, Y., Guo, M., Mahnke, K., Rivera, M., Ravetch, J.V., Steinman, R.M., and Nussenzweig, M.C. (2001). Dendritic cells induce peripheral T cell unresponsiveness under steady state conditions in vivo. The Journal of experimental medicine *194*, 769-779.

Hegde, R., Srinivasula, S.M., Ahmad, M., Fernandes-Alnemri, T., and Alnemri, E.S. (1998). Blk, a BH3-containing mouse protein that interacts with Bcl-2 and Bcl-xL, is a potent death agonist. The Journal of biological chemistry *273*, 7783-7786.

Heil, F., Hemmi, H., Hochrein, H., Ampenberger, F., Kirschning, C., Akira, S., Lipford, G., Wagner, H., and Bauer, S. (2004). Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8. Science (New York, NY *303*, 1526-1529.

Henri, S., Vremec, D., Kamath, A., Waithman, J., Williams, S., Benoist, C., Burnham, K., Saeland, S., Handman, E., and Shortman, K. (2001). The dendritic cell populations of mouse lymph nodes. J Immunol *167*, 741-748.

Hernandez, J., Aung, S., Redmond, W.L., and Sherman, L.A. (2001). Phenotypic and functional analysis of CD8(+) T cells undergoing peripheral deletion in response to cross-presentation of self-antigen. The Journal of experimental medicine *194*, 707-717.

Hertz, C.J., Wu, Q., Porter, E.M., Zhang, Y.J., Weismuller, K.H., Godowski, P.J., Ganz, T., Randell, S.H., and Modlin, R.L. (2003). Activation of Toll-like receptor 2 on human tracheobronchial epithelial cells induces the antimicrobial peptide human beta defensin-2. J Immunol *171*, 6820-6826.

Heusel, J.W., Wesselschmidt, R.L., Shresta, S., Russell, J.H., and Ley, T.J. (1994). Cytotoxic lymphocytes require granzyme B for the rapid induction of DNA fragmentation and apoptosis in allogeneic target cells. Cell *76*, 977-987.

Hildeman, D.A., Zhu, Y., Mitchell, T.C., Bouillet, P., Strasser, A., Kappler, J., and Marrack, P. (2002). Activated T cell death in vivo mediated by proapoptotic bcl-2 family member bim. Immunity *16*, 759-767.

Hill, A.V. (1999). Immunogenetics. Defence by diversity. Nature *398*, 668-669.

Hilleman, M.R. (2004). Strategies and mechanisms for host and pathogen survival in acute and persistent viral infections. Proceedings of the National Academy of Sciences of the United States of America *101 Suppl 2*, 14560-14566.

Hislop, A.D., Annels, N.E., Gudgeon, N.H., Leese, A.M., and Rickinson, A.B. (2002). Epitope-specific evolution of human CD8(+) T cell responses from primary to persistent phases of Epstein-Barr virus infection. The Journal of experimental medicine *195*, 893-905.

Hobohm, U., and Meyerhans, A. (1993). A pattern search method for putative anchor residues in T cell epitopes. European journal of immunology *23*, 1271-1276.

Hochrein, H., Shortman, K., Vremec, D., Scott, B., Hertzog, P., and O'Keeffe, M. (2001). Differential production of IL-12, IFN-alpha, and IFN-gamma by mouse dendritic cell subsets. J Immunol *166*, 5448-5455.

Houde, M., Bertholet, S., Gagnon, E., Brunet, S., Goyette, G., Laplante, A., Princiotta, M.F., Thibault, P., Sacks, D., and Desjardins, M. (2003). Phagosomes are competent organelles for antigen cross-presentation. Nature *425*, 402-406.

Hsu, H., Xiong, J., and Goeddel, D.V. (1995). The TNF receptor 1-associated protein TRADD signals cell death and NF-kappa B activation. Cell *81*, 495-504.

Hsu, S.Y., Kaipia, A., McGee, E., Lomeli, M., and Hsueh, A.J. (1997). Bok is a pro-apoptotic Bcl-2 protein with restricted expression in reproductive tissues and heterodimerizes with selective anti-apoptotic Bcl-2 family members. Proceedings of the National Academy of Sciences of the United States of America *94*, 12401-12406.

Hsu, Y.S., Chien, R.N., Yeh, C.T., Sheen, I.S., Chiou, H.Y., Chu, C.M., and Liaw, Y.F. (2002). Long-term outcome after spontaneous HBeAg seroconversion in patients with chronic hepatitis B. Hepatology (Baltimore, Md *35*, 1522-1527.

Hu, Z., Zhang, Z., Doo, E., Coux, O., Goldberg, A.L., and Liang, T.J. (1999). Hepatitis B virus X protein is both a substrate and a potential inhibitor of the proteasome complex. Journal of virology *73*, 7231-7240.

Huang, D.C., and Strasser, A. (2000). BH3-Only proteins-essential initiators of apoptotic cell death. Cell *103*, 839-842.

Huster, K.M., Koffler, M., Stemberger, C., Schiemann, M., Wagner, H., and Busch, D.H. (2006). Unidirectional development of CD8+ central memory T cells into protective Listeria-specific effector memory T cells. European journal of immunology *36*, 1453-1464.

Hyodo, N., Nakamura, I., and Imawari, M. (2004). Hepatitis B core antigen stimulates interleukin-10 secretion by both T cells and monocytes from peripheral

blood of patients with chronic hepatitis B virus infection. Clinical and experimental immunology *135*, 462-466.

Iannello, A., Debbeche, O., Martin, E., Attalah, L.H., Samarani, S., and Ahmad, A. (2006). Viral strategies for evading antiviral cellular immune responses of the host. J Leukoc Biol *79*, 16-35.

Iezzi, G., Karjalainen, K., and Lanzavecchia, A. (1998). The duration of antigenic stimulation determines the fate of naive and effector T cells. Immunity *8*, 89-95.

Imaizumi, K., Tsuda, M., Imai, Y., Wanaka, A., Takagi, T., and Tohyama, M. (1997). Molecular cloning of a novel polypeptide, DP5, induced during programmed neuronal death. The Journal of biological chemistry *272*, 18842-18848.

Iyoda, T., Shimoyama, S., Liu, K., Omatsu, Y., Akiyama, Y., Maeda, Y., Takahara, K., Steinman, R.M., and Inaba, K. (2002). The CD8+ dendritic cell subset selectively endocytoses dying cells in culture and in vivo. The Journal of experimental medicine *195*, 1289-1302.

Janeway, C.A., Jr., Dianzani, U., Portoles, P., Rath, S., Reich, E.P., Rojo, J., Yagi, J., and Murphy, D.B. (1989). Cross-linking and conformational change in T-cell receptors: role in activation and in repertoire selection. Cold Spring Harbor symposia on quantitative biology *54 Pt 2*, 657-666.

Jarosch, E., Geiss-Friedlander, R., Meusser, B., Walter, J., and Sommer, T. (2002). Protein dislocation from the endoplasmic reticulum--pulling out the suspect. Traffic (Copenhagen, Denmark) *3*, 530-536.

Jenkins, M.K., Taylor, P.S., Norton, S.D., and Urdahl, K.B. (1991). CD28 delivers a costimulatory signal involved in antigen-specific IL-2 production by human T cells. J Immunol *147*, 2461-2466.

Ji, M.J., Su, C., Wu, H.W., Zhu, X., Cai, X.P., Li, C.L., Li, G.F., Wang, Y., Zhang, Z.S., and Wu, G.L. (2003). Gene expression profile of CD4+ T cells reveals an

interferon signaling suppression associated with progression of experimental Schistosoma japonicum infection. Cellular immunology *224*, 55-62.

Jin, Y., Shih, W.K., and Berkower, I. (1988). Human T cell response to the surface antigen of hepatitis B virus (HBsAg). Endosomal and nonendosomal processing pathways are accessible to both endogenous and exogenous antigen. The Journal of experimental medicine *168*, 293-306.

Jones, B.C., Logsdon, N.J., Josephson, K., Cook, J., Barry, P.A., and Walter, M.R. (2002). Crystal structure of human cytomegalovirus IL-10 bound to soluble human IL-10R1. Proceedings of the National Academy of Sciences of the United States of America *99*, 9404-9409.

Jung, M.C., Hartmann, B., Gerlach, J.T., Diepolder, H., Gruber, R., Schraut, W., Gruner, N., Zachoval, R., Hoffmann, R., Santantonio, T., *et al.* (1999). Virus-specific lymphokine production differs quantitatively but not qualitatively in acute and chronic hepatitis B infection. Virology *261*, 165-172.

Jung, M.C., Spengler, U., Schraut, W., Hoffmann, R., Zachoval, R., Eisenburg, J., Eichenlaub, D., Riethmuller, G., Paumgartner, G., Ziegler-Heitbrock, H.W., *et al.* (1991). Hepatitis B virus antigen-specific T-cell activation in patients with acute and chronic hepatitis B. Journal of hepatology *13*, 310-317.

Jung, S., Unutmaz, D., Wong, P., Sano, G., De los Santos, K., Sparwasser, T., Wu, S., Vuthoori, S., Ko, K., Zavala, F., *et al.* (2002). In vivo depletion of CD11c(+) dendritic cells abrogates priming of CD8(+) T cells by exogenous cell-associated antigens. Immunity *17*, 211-220.

Jung, T., Schauer, U., Heusser, C., Neumann, C., and Rieger, C. (1993). Detection of intracellular cytokines by flow cytometry. Journal of immunological methods *159*, 197-207.

Kadowaki, N., Ho, S., Antonenko, S., Malefyt, R.W., Kastelein, R.A., Bazan, F., and Liu, Y.J. (2001). Subsets of human dendritic cell precursors express different toll-like

receptors and respond to different microbial antigens. The Journal of experimental medicine *194*, 863-869.

Kaech, S.M., and Ahmed, R. (2001). Memory CD8+ T cell differentiation: initial antigen encounter triggers a developmental program in naive cells. Nature immunology *2*, 415-422.

Kaech, S.M., Tan, J.T., Wherry, E.J., Konieczny, B.T., Surh, C.D., and Ahmed, R. (2003). Selective expression of the interleukin 7 receptor identifies effector CD8 T cells that give rise to long-lived memory cells. Nature immunology *4*, 1191-1198.

Kaech, S.M., Wherry, E.J., and Ahmed, R. (2002). Effector and memory T-cell differentiation: implications for vaccine development. Nature reviews *2*, 251-262.

Kagnoff, M.F., and Eckmann, L. (1997). Epithelial cells as sensors for microbial infection. The Journal of clinical investigation *100*, 6-10.

Kakimi, K., Guidotti, L.G., Koezuka, Y., and Chisari, F.V. (2000). Natural killer T cell activation inhibits hepatitis B virus replication in vivo. The Journal of experimental medicine *192*, 921-930.

Kakimi, K., Isogawa, M., Chung, J., Sette, A., and Chisari, F.V. (2002). Immunogenicity and tolerogenicity of hepatitis B virus structural and nonstructural proteins: implications for immunotherapy of persistent viral infections. Journal of virology *76*, 8609-8620.

Kalams, S.A., and Walker, B.D. (1998). The critical need for CD4 help in maintaining effective cytotoxic T lymphocyte responses. The Journal of experimental medicine *188*, 2199-2204.

Kanto, T., Hayashi, N., Takehara, T., Tatsumi, T., Kuzushita, N., Ito, A., Sasaki, Y., Kasahara, A., and Hori, M. (1999). Impaired allostimulatory capacity of peripheral blood dendritic cells recovered from hepatitis C virus-infected individuals. J Immunol *162*, 5584-5591.

Kao, C., Daniels, M.A., and Jameson, S.C. (2005). Loss of CD8 and TCR binding to Class I MHC ligands following T cell activation. International immunology *17*, 1607-1617.

Kapp, J.A., Kapp, L.M., and McKenna, K.C. (2004). Gammadelta T cells play an essential role in several forms of tolerance. Immunologic research *29*, 93-102.

Kato, H., Sato, S., Yoneyama, M., Yamamoto, M., Uematsu, S., Matsui, K., Tsujimura, T., Takeda, K., Fujita, T., Takeuchi, O., *et al.* (2005). Cell type-specific involvement of RIG-I in antiviral response. Immunity *23*, 19-28.

Kato, H., Takeuchi, O., Sato, S., Yoneyama, M., Yamamoto, M., Matsui, K., Uematsu, S., Jung, A., Kawai, T., Ishii, K.J., *et al.* (2006). Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses. Nature *441*, 101-105.

Katze, M.G., He, Y., and Gale, M., Jr. (2002). Viruses and interferon: a fight for supremacy. Nature reviews *2*, 675-687.

Kawai, T., and Akira, S. (2006). TLR signaling. Cell death and differentiation *13*, 816-825.

Ke, N., Godzik, A., and Reed, J.C. (2001). Bcl-B, a novel Bcl-2 family member that differentially binds and regulates Bax and Bak. The Journal of biological chemistry *276*, 12481-12484.

Ke, Y., and Kapp, J.A. (1996). Exogenous antigens gain access to the major histocompatibility complex class I processing pathway in B cells by receptor-mediated uptake. The Journal of experimental medicine *184*, 1179-1184.

Kedl, R.M., Kappler, J.W., and Marrack, P. (2003). Epitope dominance, competition and T cell affinity maturation. Current opinion in immunology *15*, 120-127.

Kekow, J., Wachsman, W., McCutchan, J.A., Cronin, M., Carson, D.A., and Lotz, M. (1990). Transforming growth factor beta and noncytopathic mechanisms of immunodeficiency in human immunodeficiency virus infection. Proceedings of the National Academy of Sciences of the United States of America *87*, 8321-8325.

Kelekar, A., and Thompson, C.B. (1998). Bcl-2-family proteins: the role of the BH3 domain in apoptosis. Trends in cell biology *8*, 324-330.

Kersh, E.N., Kaech, S.M., Onami, T.M., Moran, M., Wherry, E.J., Miceli, M.C., and Ahmed, R. (2003). TCR signal transduction in antigen-specific memory CD8 T cells. J Immunol *170*, 5455-5463.

Khanna, K.M., Lepisto, A.J., Decman, V., and Hendricks, R.L. (2004). Immune control of herpes simplex virus during latency. Current opinion in immunology *16*, 463-469.

Kiefer, M.C., Brauer, M.J., Powers, V.C., Wu, J.J., Umansky, S.R., Tomei, L.D., and Barr, P.J. (1995). Modulation of apoptosis by the widely distributed Bcl-2 homologue Bak. Nature *374*, 736-739.

Kim, C.Y., and Tilles, J.G. (1973). Purification and biophysical characterization of hepatitis B antigen. The Journal of clinical investigation *52*, 1176-1186.

Klenerman, P., and Hill, A. (2005). T cells and viral persistence: lessons from diverse infections. Nature immunology *6*, 873-879.

Klenerman, P., and Ludewig, B. (2006). Virus scores a perfect 10. Nature medicine *12*, 1246-1248.

Klenerman, P., Rowland-Jones, S., McAdam, S., Edwards, J., Daenke, S., Lalloo, D., Koppe, B., Rosenberg, W., Boyd, D., Edwards, A., *et al.* (1994). Cytotoxic T-cell activity antagonized by naturally occurring HIV-1 Gag variants. Nature *369*, 403-407.

Knolle, P.A., and Gerken, G. (2000). Local control of the immune response in the liver. Immunological reviews *174*, 21-34.

Knolle, P.A., Schmitt, E., Jin, S., Germann, T., Duchmann, R., Hegenbarth, S., Gerken, G., and Lohse, A.W. (1999). Induction of cytokine production in naive CD4(+) T cells by antigen-presenting murine liver sinusoidal endothelial cells but failure to induce differentiation toward Th1 cells. Gastroenterology *116*, 1428-1440.

Knolle, P.A., Uhrig, A., Hegenbarth, S., Loser, E., Schmitt, E., Gerken, G., and Lohse, A.W. (1998). IL-10 down-regulates T cell activation by antigen-presenting liver sinusoidal endothelial cells through decreased antigen uptake via the mannose receptor and lowered surface expression of accessory molecules. Clinical and experimental immunology *114*, 427-433.

Kotenko, S.V., Saccani, S., Izotova, L.S., Mirochnitchenko, O.V., and Pestka, S. (2000). Human cytomegalovirus harbors its own unique IL-10 homolog (cmvIL-10). Proceedings of the National Academy of Sciences of the United States of America *97*, 1695-1700.

Koup, R.A. (1994). Virus escape from CTL recognition. The Journal of experimental medicine *180*, 779-782.

Kozopas, K.M., Yang, T., Buchan, H.L., Zhou, P., and Craig, R.W. (1993). MCL1, a gene expressed in programmed myeloid cell differentiation, has sequence similarity to BCL2. Proceedings of the National Academy of Sciences of the United States of America *90*, 3516-3520.

Krajewski, S., Tanaka, S., Takayama, S., Schibler, M.J., Fenton, W., and Reed, J.C. (1993). Investigation of the subcellular distribution of the bcl-2 oncoprotein: residence in the nuclear envelope, endoplasmic reticulum, and outer mitochondrial membranes. Cancer research *53*, 4701-4714.

Krammer, P.H., Arnold, R., and Lavrik, I.N. (2007). Life and death in peripheral T cells. Nature reviews *7*, 532-542.

Kramvis, A., and Kew, M.C. (1999). The core promoter of hepatitis B virus. Journal of viral hepatitis *6*, 415-427.

Kreuwel, H.T., Aung, S., Silao, C., and Sherman, L.A. (2002). Memory CD8(+) T cells undergo peripheral tolerance. Immunity *17*, 73-81.

Kroczek, R.A., Mages, H.W., and Hutloff, A. (2004). Emerging paradigms of T-cell co-stimulation. Current opinion in immunology *16*, 321-327.

Kroemer, G. (1997). The proto-oncogene Bcl-2 and its role in regulating apoptosis. Nature medicine *3*, 614-620.

Kuhns, M.S., Davis, M.M., and Garcia, K.C. (2006). Deconstructing the form and function of the TCR/CD3 complex. Immunity *24*, 133-139.

Kumar, S. (2007). Caspase function in programmed cell death. Cell death and differentiation *14*, 32-43.

Kurts, C., Heath, W.R., Carbone, F.R., Allison, J., Miller, J.F., and Kosaka, H. (1996). Constitutive class I-restricted exogenous presentation of self antigens in vivo. The Journal of experimental medicine *184*, 923-930.

Kurts, C., Kosaka, H., Carbone, F.R., Miller, J.F., and Heath, W.R. (1997). Class I-restricted cross-presentation of exogenous self-antigens leads to deletion of autoreactive CD8(+) T cells. The Journal of experimental medicine *186*, 239-245.

Kyewski, B., and Klein, L. (2006). A central role for central tolerance. Annual review of immunology *24*, 571-606.

La Gruta, N.L., Kedzierska, K., Pang, K., Webby, R., Davenport, M., Chen, W., Turner, S.J., and Doherty, P.C. (2006). A virus-specific CD8+ T cell immunodominance hierarchy determined by antigen dose and precursor frequencies. Proceedings of the National Academy of Sciences of the United States of America *103*, 994-999.

Lanier, L.L. (2005). NK cell recognition. Annual review of immunology *23*, 225-274.

Lanzavecchia, A., and Sallusto, F. (2001). Antigen decoding by T lymphocytes: from synapses to fate determination. Nature immunology *2*, 487-492.

Larsson, K., Lindstedt, M., and Borrebaeck, C.A. (2006). Functional and transcriptional profiling of MUTZ-3, a myeloid cell line acting as a model for dendritic cells. Immunology *117*, 156-166.

Larsson, M., Messmer, D., Somersan, S., Fonteneau, J.F., Donahoe, S.M., Lee, M., Dunbar, P.R., Cerundolo, V., Julkunen, I., Nixon, D.F., *et al.* (2000). Requirement of mature dendritic cells for efficient activation of influenza A-specific memory CD8+ T cells. J Immunol *165*, 1182-1190.

Le Bon, A., Etchart, N., Rossmann, C., Ashton, M., Hou, S., Gewert, D., Borrow, P., and Tough, D.F. (2003). Cross-priming of CD8+ T cells stimulated by virus-induced type I interferon. Nature immunology *4*, 1009-1015.

Le Gall, S., Erdtmann, L., Benichou, S., Berlioz-Torrent, C., Liu, L., Benarous, R., Heard, J.M., and Schwartz, O. (1998). Nef interacts with the mu subunit of clathrin adaptor complexes and reveals a cryptic sorting signal in MHC I molecules. Immunity *8*, 483-495.

Lee, C.K., Rao, D.T., Gertner, R., Gimeno, R., Frey, A.B., and Levy, D.E. (2000). Distinct requirements for IFNs and STAT1 in NK cell function. J Immunol *165*, 3571-3577.

Lehner, P.J., Karttunen, J.T., Wilkinson, G.W., and Cresswell, P. (1997). The human cytomegalovirus US6 glycoprotein inhibits transporter associated with antigen processing-dependent peptide translocation. Proceedings of the National Academy of Sciences of the United States of America *94*, 6904-6909.

Lenardo, M., Chan, K.M., Hornung, F., McFarland, H., Siegel, R., Wang, J., and Zheng, L. (1999). Mature T lymphocyte apoptosis--immune regulation in a dynamic and unpredictable antigenic environment. Annual review of immunology *17*, 221-253.

Lessard, J., Faubert, A., and Sauvageau, G. (2004). Genetic programs regulating HSC specification, maintenance and expansion. Oncogene *23*, 7199-7209.

Levitskaya, J., Sharipo, A., Leonchiks, A., Ciechanover, A., and Masucci, M.G. (1997). Inhibition of ubiquitin/proteasome-dependent protein degradation by the Gly-Ala repeat domain of the Epstein-Barr virus nuclear antigen 1. Proceedings of the National Academy of Sciences of the United States of America *94*, 12616-12621.

Li, L., Iwamoto, Y., Berezovskaya, A., and Boussiotis, V.A. (2006). A pathway regulated by cell cycle inhibitor p27Kip1 and checkpoint inhibitor Smad3 is involved in the induction of T cell tolerance. Nature immunology *7*, 1157-1165.

Li, W., Kashiwamura, S.I., Ueda, H., Sekiyama, A., and Okamura, H. (2007). Protection of CD8+ T cells from activation-induced cell death by IL-18. J Leukoc Biol.

Lichterfeld, M., Yu, X.G., Waring, M.T., Mui, S.K., Johnston, M.N., Cohen, D., Addo, M.M., Zaunders, J., Alter, G., Pae, E., *et al.* (2004). HIV-1-specific cytotoxicity is preferentially mediated by a subset of CD8(+) T cells producing both interferon-gamma and tumor necrosis factor-alpha. Blood *104*, 487-494.

Lieberman, J. (2003). The ABCs of granule-mediated cytotoxicity: new weapons in the arsenal. Nature reviews *3*, 361-370.

Lieberman, J., Manjunath, N., and Shankar, P. (2002). Avoiding the kiss of death: how HIV and other chronic viruses survive. Current opinion in immunology *14*, 478-486.

Lim, D.G., Bieganowska Bourcier, K., Freeman, G.J., and Hafler, D.A. (2000). Examination of CD8+ T cell function in humans using MHC class I tetramers: similar cytotoxicity but variable proliferation and cytokine production among different clonal CD8+ T cells specific to a single viral epitope. J Immunol *165*, 6214-6220.

Lim, J.S., Kim, S., Lee, H.G., Lee, K.Y., Kwon, T.J., and Kim, K. (1996). Selection of peptides that bind to the HLA-A2.1 molecule by molecular modelling. Molecular immunology *33*, 221-230.

Limmer, A., Ohl, J., Kurts, C., Ljunggren, H.G., Reiss, Y., Groettrup, M., Momburg, F., Arnold, B., and Knolle, P.A. (2000). Efficient presentation of exogenous antigen by liver endothelial cells to CD8+ T cells results in antigen-specific T-cell tolerance. Nature medicine *6*, 1348-1354.

Lin, E.Y., Orlofsky, A., Wang, H.G., Reed, J.C., and Prystowsky, M.B. (1996). A1, a Bcl-2 family member, prolongs cell survival and permits myeloid differentiation. Blood *87*, 983-992.

Lithgow, T., van Driel, R., Bertram, J.F., and Strasser, A. (1994). The protein product of the oncogene bcl-2 is a component of the nuclear envelope, the endoplasmic reticulum, and the outer mitochondrial membrane. Cell Growth Differ *5*, 411-417.

Liu, J., Rivas, F.V., Wohlschlegel, J., Yates, J.R., 3rd, Parker, R., and Hannon, G.J. (2005a). A role for the P-body component GW182 in microRNA function. Nature cell biology *7*, 1261-1266.

Liu, J., Valencia-Sanchez, M.A., Hannon, G.J., and Parker, R. (2005b). MicroRNA-dependent localization of targeted mRNAs to mammalian P-bodies. Nature cell biology *7*, 719-723.

Liu, L., Tran, E., Zhao, Y., Huang, Y., Flavell, R., and Lu, B. (2005c). Gadd45 beta and Gadd45 gamma are critical for regulating autoimmunity. The Journal of experimental medicine *202*, 1341-1347.

Ljunggren, H.G., and Karre, K. (1985). Host resistance directed selectively against H-2-deficient lymphoma variants. Analysis of the mechanism. The Journal of experimental medicine *162*, 1745-1759.

Locarnini, S. (2004). Molecular virology of hepatitis B virus. Seminars in liver disease *24 Suppl 1*, 3-10.

Locarnini, S., McMillan, J., and Bartholomeusz, A. (2003). The hepatitis B virus and common mutants. Seminars in liver disease *23*, 5-20.

Lohr, H.F., Weber, W., Schlaak, J., Goergen, B., Meyer zum Buschenfelde, K.H., and Gerken, G. (1995). Proliferative response of CD4+ T cells and hepatitis B virus clearance in chronic hepatitis with or without hepatitis B e-minus hepatitis B virus mutants. Hepatology (Baltimore, Md *22*, 61-68.

Lok, A.S., Lai, C.L., Wu, P.C., Leung, E.K., and Lam, T.S. (1987). Spontaneous hepatitis B e antigen to antibody seroconversion and reversion in Chinese patients with chronic hepatitis B virus infection. Gastroenterology *92*, 1839-1843.

Lopes, A.R., Jaye, A., Dorrell, L., Sabally, S., Alabi, A., Jones, N.A., Flower, D.R., De Groot, A., Newton, P., Lascar, R.M., *et al.* (2003). Greater CD8+ TCR heterogeneity and functional flexibility in HIV-2 compared to HIV-1 infection. J Immunol *171*, 307-316.

Lord, J.D., McIntosh, B.C., Greenberg, P.D., and Nelson, B.H. (2000). The IL-2 receptor promotes lymphocyte proliferation and induction of the c-myc, bcl-2, and bcl-x genes through the trans-activation domain of Stat5. J Immunol *164*, 2533-2541.

Lotz, M., Kekow, J., and Carson, D.A. (1990). Transforming growth factor-beta and cellular immune responses in synovial fluids. J Immunol *144*, 4189-4194.

Lund, J.M., Alexopoulou, L., Sato, A., Karow, M., Adams, N.C., Gale, N.W., Iwasaki, A., and Flavell, R.A. (2004). Recognition of single-stranded RNA viruses by Toll-like receptor 7. Proceedings of the National Academy of Sciences of the United States of America *101*, 5598-5603.

Lund, R., Ahlfors, H., Kainonen, E., Lahesmaa, A.M., Dixon, C., and Lahesmaa, R. (2005). Identification of genes involved in the initiation of human Th1 or Th2 cell commitment. European journal of immunology *35*, 3307-3319.

Macian, F., Im, S.H., Garcia-Cozar, F.J., and Rao, A. (2004). T-cell anergy. Current opinion in immunology *16*, 209-216.

Maecker, H.T., and Maino, V.C. (2004). Analyzing T-cell responses to cytomegalovirus by cytokine flow cytometry. Human immunology *65*, 493-499.

Maile, R., Siler, C.A., Kerry, S.E., Midkiff, K.E., Collins, E.J., and Frelinger, J.A. (2005). Peripheral "CD8 tuning" dynamically modulates the size and responsiveness of an antigen-specific T cell pool in vivo. J Immunol *174*, 619-627.

Maini, M.K., and Bertoletti, A. (2000). How can the cellular immune response control hepatitis B virus replication? Journal of viral hepatitis *7*, 321-326.

Maini, M.K., Boni, C., Lee, C.K., Larrubia, J.R., Reignat, S., Ogg, G.S., King, A.S., Herberg, J., Gilson, R., Alisa, A., *et al.* (2000a). The role of virus-specific CD8(+) cells in liver damage and viral control during persistent hepatitis B virus infection. The Journal of experimental medicine *191*, 1269-1280.

Maini, M.K., Boni, C., Ogg, G.S., King, A.S., Reignat, S., Lee, C.K., Larrubia, J.R., Webster, G.J., McMichael, A.J., Ferrari, C., *et al.* (1999). Direct ex vivo analysis of hepatitis B virus-specific CD8(+) T cells associated with the control of infection. Gastroenterology *117*, 1386-1396.

Maini, M.K., Reignat, S., Boni, C., Ogg, G.S., King, A.S., Malacarne, F., Webster, G.J., and Bertoletti, A. (2000b). T cell receptor usage of virus-specific CD8 cells and recognition of viral mutations during acute and persistent hepatitis B virus infection. European journal of immunology *30*, 3067-3078.

Malmassari, S., Lone, Y.C., Zhang, M., Transy, C., and Michel, M.L. (2005). In vivo hierarchy of immunodominant and subdominant HLA-A*0201-restricted T-cell epitopes of HBx antigen of hepatitis B virus. Microbes and infection / Institut Pasteur *7*, 626-634.

Maloy, K.J., and Powrie, F. (2001). Regulatory T cells in the control of immune pathology. Nature immunology *2*, 816-822.

Mangeat, B., Turelli, P., Caron, G., Friedli, M., Perrin, L., and Trono, D. (2003). Broad antiretroviral defence by human APOBEC3G through lethal editing of nascent reverse transcripts. Nature *424*, 99-103.

Marani, M., Tenev, T., Hancock, D., Downward, J., and Lemoine, N.R. (2002). Identification of novel isoforms of the BH3 domain protein Bim which directly activate Bax to trigger apoptosis. Molecular and cellular biology *22*, 3577-3589.

Maraskovsky, E., O'Reilly, L.A., Teepe, M., Corcoran, L.M., Peschon, J.J., and Strasser, A. (1997). Bcl-2 can rescue T lymphocyte development in interleukin-7 receptor-deficient mice but not in mutant rag-1-/- mice. Cell *89*, 1011-1019.

Marie, I., Durbin, J.E., and Levy, D.E. (1998). Differential viral induction of distinct interferon-alpha genes by positive feedback through interferon regulatory factor-7. The EMBO journal *17*, 6660-6669.

Marinos, G., Torre, F., Chokshi, S., Hussain, M., Clarke, B.E., Rowlands, D.J., Eddleston, A.L., Naoumov, N.V., and Williams, R. (1995). Induction of T-helper cell response to hepatitis B core antigen in chronic hepatitis B: a major factor in activation of the host immune response to the hepatitis B virus. Hepatology (Baltimore, Md *22*, 1040-1049.

Maris, C.H., Chappell, C.P., and Jacob, J. (2007). Interleukin-10 plays an early role in generating virus-specific T cell anergy. BMC immunology *8*, 8.

Marsland, B.J., Battig, P., Bauer, M., Ruedl, C., Lassing, U., Beerli, R.R., Dietmeier, K., Ivanova, L., Pfister, T., Vogt, L., *et al.* (2005). CCL19 and CCL21 induce a potent proinflammatory differentiation program in licensed dendritic cells. Immunity *22*, 493-505.

Martin, D.A., Siegel, R.M., Zheng, L., and Lenardo, M.J. (1998). Membrane oligomerization and cleavage activates the caspase-8 (FLICE/MACHalpha1) death signal. The Journal of biological chemistry *273*, 4345-4349.

Martino, A., Holmes, J.H.t., Lord, J.D., Moon, J.J., and Nelson, B.H. (2001). Stat5 and Sp1 regulate transcription of the cyclin D2 gene in response to IL-2. J Immunol *166*, 1723-1729.

Masopust, D., Vezys, V., Marzo, A.L., and Lefrancois, L. (2001). Preferential localization of effector memory cells in nonlymphoid tissue. Science (New York, NY *291*, 2413-2417.

Mast, E.E., Mahoney, F.J., Alter, M.J., and Margolis, H.S. (1998). Progress toward elimination of hepatitis B virus transmission in the United States. Vaccine *16 Suppl*, S48-51.

Masterson, A.J., Sombroek, C.C., De Gruijl, T.D., Graus, Y.M., van der Vliet, H.J., Lougheed, S.M., van den Eertwegh, A.J., Pinedo, H.M., and Scheper, R.J. (2002). MUTZ-3, a human cell line model for the cytokine-induced differentiation of dendritic cells from CD34+ precursors. Blood *100*, 701-703.

Matlack, K.E., Mothes, W., and Rapoport, T.A. (1998). Protein translocation: tunnel vision. Cell *92*, 381-390.

Matyszak, M.K., Citterio, S., Rescigno, M., and Ricciardi-Castagnoli, P. (2000). Differential effects of corticosteroids during different stages of dendritic cell maturation. European journal of immunology *30*, 1233-1242.

Matzinger, P. (1994). Tolerance, danger, and the extended family. Annual review of immunology *12*, 991-1045.

Matzinger, P. (2002). The danger model: a renewed sense of self. Science (New York, NY *296*, 301-305.

McMahon, B.J. (2005). Epidemiology and natural history of hepatitis B. Seminars in liver disease *25 Suppl 1*, 3-8.

McMahon, B.J., Alward, W.L., Hall, D.B., Heyward, W.L., Bender, T.R., Francis, D.P., and Maynard, J.E. (1985). Acute hepatitis B virus infection: relation of age to the clinical expression of disease and subsequent development of the carrier state. The Journal of infectious diseases *151*, 599-603.

Medzhitov, R., and Janeway, C.A., Jr. (1997). Innate immunity: impact on the adaptive immune response. Current opinion in immunology *9*, 4-9.

Medzhitov, R., and Janeway, C.A., Jr. (2000). How does the immune system distinguish self from nonself? Seminars in immunology *12*, 185-188; discussion 257-344.

Mercado, R., Vijh, S., Allen, S.E., Kerksiek, K., Pilip, I.M., and Pamer, E.G. (2000). Early programming of T cell populations responding to bacterial infection. J Immunol *165*, 6833-6839.

Merritt, C., Enslen, H., Diehl, N., Conze, D., Davis, R.J., and Rincon, M. (2000). Activation of p38 mitogen-activated protein kinase in vivo selectively induces apoptosis of CD8(+) but not CD4(+) T cells. Molecular and cellular biology *20*, 936-946.

Meyaard, L., Otto, S.A., Jonker, R.R., Mijnster, M.J., Keet, R.P., and Miedema, F. (1992). Programmed death of T cells in HIV-1 infection. Science (New York, NY *257*, 217-219.

Meylan, E., Curran, J., Hofmann, K., Moradpour, D., Binder, M., Bartenschlager, R., and Tschopp, J. (2005). Cardif is an adaptor protein in the RIG-I antiviral pathway and is targeted by hepatitis C virus. Nature *437*, 1167-1172.

Meylan, E., and Tschopp, J. (2006). Toll-like receptors and RNA helicases: two parallel ways to trigger antiviral responses. Molecular cell *22*, 561-569.

Michallet, M.C., Saltel, F., Flacher, M., Revillard, J.P., and Genestier, L. (2004). Cathepsin-dependent apoptosis triggered by supraoptimal activation of T lymphocytes: a possible mechanism of high dose tolerance. J Immunol *172*, 5405-5414.

Michallet, M.C., Saltel, F., Preville, X., Flacher, M., Revillard, J.P., and Genestier, L. (2003). Cathepsin-B-dependent apoptosis triggered by antithymocyte globulins: a novel mechanism of T-cell depletion. Blood *102*, 3719-3726.

Milhas, D., Cuvillier, O., Therville, N., Clave, P., Thomsen, M., Levade, T., Benoist, H., and Segui, B. (2005). Caspase-10 triggers Bid cleavage and caspase cascade activation in FasL-induced apoptosis. The Journal of biological chemistry *280*, 19836-19842.

Milich, D.R. (1997). Influence of T-helper cell subsets and crossregulation in hepatitis B virus infection. Journal of viral hepatitis *4 Suppl 2*, 48-59.

Milich, D.R., Chen, M.K., Hughes, J.L., and Jones, J.E. (1998). The secreted hepatitis B precore antigen can modulate the immune response to the nucleocapsid: a mechanism for persistence. J Immunol *160*, 2013-2021.

Milich, D.R., Jones, J.E., Hughes, J.L., Price, J., Raney, A.K., and McLachlan, A. (1990). Is a function of the secreted hepatitis B e antigen to induce immunologic tolerance in utero? Proceedings of the National Academy of Sciences of the United States of America *87*, 6599-6603.

Milich, D.R., and McLachlan, A. (1986). The nucleocapsid of hepatitis B virus is both a T-cell-independent and a T-cell-dependent antigen. Science (New York, NY *234*, 1398-1401.

Milich, D.R., McLachlan, A., Moriarty, A., and Thornton, G.B. (1987). Immune response to hepatitis B virus core antigen (HBcAg): localization of T cell recognition sites within HBcAg/HBeAg. J Immunol *139*, 1223-1231.

Milich, D.R., Thornton, G.B., Neurath, A.R., Kent, S.B., Michel, M.L., Tiollais, P., and Chisari, F.V. (1985). Enhanced immunogenicity of the pre-S region of hepatitis B surface antigen. Science (New York, NY *228*, 1195-1199.

Miller, M.J., Hejazi, A.S., Wei, S.H., Cahalan, M.D., and Parker, I. (2004). T cell repertoire scanning is promoted by dynamic dendritic cell behavior and random T cell motility in the lymph node. Proceedings of the National Academy of Sciences of the United States of America *101*, 998-1003.

Missale, G., Redeker, A., Person, J., Fowler, P., Guilhot, S., Schlicht, H.J., Ferrari, C., and Chisari, F.V. (1993). HLA-A31- and HLA-Aw68-restricted cytotoxic T cell responses to a single hepatitis B virus nucleocapsid epitope during acute viral hepatitis. The Journal of experimental medicine *177*, 751-762.

Miyazaki, T., Liu, Z.J., Kawahara, A., Minami, Y., Yamada, K., Tsujimoto, Y., Barsoumian, E.L., Permutter, R.M., and Taniguchi, T. (1995). Three distinct IL-2 signaling pathways mediated by bcl-2, c-myc, and lck cooperate in hematopoietic cell proliferation. Cell *81*, 223-231.

Mizukoshi, E., Sidney, J., Livingston, B., Ghany, M., Hoofnagle, J.H., Sette, A., and Rehermann, B. (2004). Cellular immune responses to the hepatitis B virus polymerase. J Immunol *173*, 5863-5871.

Molon, B., Gri, G., Bettella, M., Gomez-Mouton, C., Lanzavecchia, A., Martinez, A.C., Manes, S., and Viola, A. (2005). T cell costimulation by chemokine receptors. Nature immunology *6*, 465-471.

Morimoto, J., Tan, X., Teague, R.M., Ohlen, C., and Greenberg, P.D. (2007). Induction of tolerance in CD8+ T cells to a transgenic autoantigen expressed in the liver does not require cross-presentation. J Immunol *178*, 6849-6860.

Moser, M., De Smedt, T., Sornasse, T., Tielemans, F., Chentoufi, A.A., Muraille, E., Van Mechelen, M., Urbain, J., and Leo, O. (1995). Glucocorticoids down-regulate dendritic cell function in vitro and in vivo. European journal of immunology *25*, 2818-2824.

Moskophidis, D., Lechner, F., Pircher, H., and Zinkernagel, R.M. (1993). Virus persistence in acutely infected immunocompetent mice by exhaustion of antiviral cytotoxic effector T cells. Nature *362*, 758-761.

Mosmann, T.R., and Coffman, R.L. (1989). TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. Annual review of immunology *7*, 145-173.

Murali-Krishna, K., Altman, J.D., Suresh, M., Sourdive, D.J., Zajac, A.J., Miller, J.D., Slansky, J., and Ahmed, R. (1998). Counting antigen-specific CD8 T cells: a reevaluation of bystander activation during viral infection. Immunity *8*, 177-187.

Murphy, K.M., and Reiner, S.L. (2002). The lineage decisions of helper T cells. Nature reviews *2*, 933-944.

Murray, J.M., Wieland, S.F., Purcell, R.H., and Chisari, F.V. (2005). Dynamics of hepatitis B virus clearance in chimpanzees. Proceedings of the National Academy of Sciences of the United States of America *102*, 17780-17785.

Muzio, M., Stockwell, B.R., Stennicke, H.R., Salvesen, G.S., and Dixit, V.M. (1998). An induced proximity model for caspase-8 activation. The Journal of biological chemistry *273*, 2926-2930.

Nagai, M., Ijichi, S., Hall, W.W., and Osame, M. (1995). Differential effect of TGF-beta 1 on the in vitro activation of HTLV-I and the proliferative response of CD8+ T lymphocytes in patients with HTLV-I-associated myelopathy (HAM/TSP). Clinical immunology and immunopathology *77*, 324-331.

Nagai, M., Kubota, R., Greten, T.F., Schneck, J.P., Leist, T.P., and Jacobson, S. (2001). Increased activated human T cell lymphotropic virus type I (HTLV-I) Tax11-19-specific memory and effector CD8+ cells in patients with HTLV-I-associated myelopathy/tropical spastic paraparesis: correlation with HTLV-I provirus load. The Journal of infectious diseases *183*, 197-205.

Nakano, H., Yanagita, M., and Gunn, M.D. (2001). CD11c(+)B220(+)Gr-1(+) cells in mouse lymph nodes and spleen display characteristics of plasmacytoid dendritic cells. The Journal of experimental medicine *194*, 1171-1178.

Nakano, K., and Vousden, K.H. (2001). PUMA, a novel proapoptotic gene, is induced by p53. Molecular cell *7*, 683-694.

Nayersina, R., Fowler, P., Guilhot, S., Missale, G., Cerny, A., Schlicht, H.J., Vitiello, A., Chesnut, R., Person, J.L., Redeker, A.G., *et al.* (1993). HLA A2 restricted cytotoxic T lymphocyte responses to multiple hepatitis B surface antigen epitopes during hepatitis B virus infection. J Immunol *150*, 4659-4671.

Nelson, G.W., Kaslow, R., and Mann, D.L. (1997). Frequency of HLA allele-specific peptide motifs in HIV-1 proteins correlates with the allele's association with relative rates of disease progression after HIV-1 infection. Proceedings of the National Academy of Sciences of the United States of America *94*, 9802-9807.

Neurath, A.R., Kent, S.B., Strick, N., and Parker, K. (1986). Identification and chemical synthesis of a host cell receptor binding site on hepatitis B virus. Cell *46*, 429-436.

Newberg, M.H., McEvers, K.J., Gorgone, D.A., Lifton, M.A., Baumeister, S.H., Veazey, R.S., Schmitz, J.E., and Letvin, N.L. (2006). Immunodomination in the evolution of dominant epitope-specific CD8+ T lymphocyte responses in simian immunodeficiency virus-infected rhesus monkeys. J Immunol *176*, 319-328.

Nguyen, K.B., Salazar-Mather, T.P., Dalod, M.Y., Van Deusen, J.B., Wei, X.Q., Liew, F.Y., Caligiuri, M.A., Durbin, J.E., and Biron, C.A. (2002a). Coordinated and distinct roles for IFN-alpha beta, IL-12, and IL-15 regulation of NK cell responses to viral infection. J Immunol *169*, 4279-4287.

Nguyen, L., Hu, D.J., Choopanya, K., Vanichseni, S., Kitayaporn, D., van Griensven, F., Mock, P.A., Kittikraisak, W., Young, N.L., Mastro, T.D., *et al.* (2002b). Genetic analysis of incident HIV-1 strains among injection drug users in Bangkok: evidence for multiple transmission clusters during a period of high incidence. Journal of acquired immune deficiency syndromes (1999) *30*, 248-256.

Nguyen, M., Millar, D.G., Yong, V.W., Korsmeyer, S.J., and Shore, G.C. (1993). Targeting of Bcl-2 to the mitochondrial outer membrane by a COOH-terminal signal anchor sequence. The Journal of biological chemistry *268*, 25265-25268.

Norbury, C.C., Malide, D., Gibbs, J.S., Bennink, J.R., and Yewdell, J.W. (2002). Visualizing priming of virus-specific CD8+ T cells by infected dendritic cells in vivo. Nature immunology *3*, 265-271.

Nurieva, R., Thomas, S., Nguyen, T., Martin-Orozco, N., Wang, Y., Kaja, M.K., Yu, X.Z., and Dong, C. (2006). T-cell tolerance or function is determined by combinatorial costimulatory signals. The EMBO journal *25*, 2623-2633.

O'Connor, L., Strasser, A., O'Reilly, L.A., Hausmann, G., Adams, J.M., Cory, S., and Huang, D.C. (1998). Bim: a novel member of the Bcl-2 family that promotes apoptosis. The EMBO journal *17*, 384-395.

O'Reilly, L.A., Cullen, L., Visvader, J., Lindeman, G.J., Print, C., Bath, M.L., Huang, D.C., and Strasser, A. (2000). The proapoptotic BH3-only protein bim is expressed in hematopoietic, epithelial, neuronal, and germ cells. The American journal of pathology *157*, 449-461.

Oda, E., Ohki, R., Murasawa, H., Nemoto, J., Shibue, T., Yamashita, T., Tokino, T., Taniguchi, T., and Tanaka, N. (2000). Noxa, a BH3-only member of the Bcl-2 family and candidate mediator of p53-induced apoptosis. Science (New York, NY *288*, 1053-1058.

Oldstone, M.B. (2006). Viral persistence: parameters, mechanisms and future predictions. Virology *344*, 111-118.

Oltvai, Z.N., Milliman, C.L., and Korsmeyer, S.J. (1993). Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death. Cell *74*, 609-619.

Omata, M., Ehata, T., Yokosuka, O., Hosoda, K., and Ohto, M. (1991). Mutations in the precore region of hepatitis B virus DNA in patients with fulminant and severe hepatitis. The New England journal of medicine *324*, 1699-1704.

Opferman, J.T., Letai, A., Beard, C., Sorcinelli, M.D., Ong, C.C., and Korsmeyer, S.J. (2003). Development and maintenance of B and T lymphocytes requires antiapoptotic MCL-1. Nature *426*, 671-676.

Paiardini, M., Cervasi, B., Albrecht, H., Muthukumar, A., Dunham, R., Gordon, S., Radziewicz, H., Piedimonte, G., Magnani, M., Montroni, M., *et al.* (2005). Loss of

CD127 expression defines an expansion of effector CD8+ T cells in HIV-infected individuals. J Immunol *174*, 2900-2909.

Palena, C., Schlom, J., and Tsang, K.Y. (2003). Differential gene expression profiles in a human T-cell line stimulated with a tumor-associated self-peptide versus an enhancer agonist peptide. Clin Cancer Res *9*, 1616-1627.

Pantaleo, G., and Harari, A. (2006). Functional signatures in antiviral T-cell immunity for monitoring virus-associated diseases. Nature reviews *6*, 417-423.

Pellegrini, M., Belz, G., Bouillet, P., and Strasser, A. (2003). Shutdown of an acute T cell immune response to viral infection is mediated by the proapoptotic Bcl-2 homology 3-only protein Bim. Proceedings of the National Academy of Sciences of the United States of America *100*, 14175-14180.

Pellegrini, M., Bouillet, P., Robati, M., Belz, G.T., Davey, G.M., and Strasser, A. (2004). Loss of Bim increases T cell production and function in interleukin 7 receptor-deficient mice. The Journal of experimental medicine *200*, 1189-1195.

Penna, A., Artini, M., Cavalli, A., Levrero, M., Bertoletti, A., Pilli, M., Chisari, F.V., Rehermann, B., Del Prete, G., Fiaccadori, F., *et al.* (1996). Long-lasting memory T cell responses following self-limited acute hepatitis B. The Journal of clinical investigation *98*, 1185-1194.

Penna, A., Chisari, F.V., Bertoletti, A., Missale, G., Fowler, P., Giuberti, T., Fiaccadori, F., and Ferrari, C. (1991). Cytotoxic T lymphocytes recognize an HLA-A2-restricted epitope within the hepatitis B virus nucleocapsid antigen. The Journal of experimental medicine *174*, 1565-1570.

Penna, A., Del Prete, G., Cavalli, A., Bertoletti, A., D'Elios, M.M., Sorrentino, R., D'Amato, M., Boni, C., Pilli, M., Fiaccadori, F., *et al.* (1997). Predominant T-helper 1 cytokine profile of hepatitis B virus nucleocapsid-specific T cells in acute self-limited hepatitis B. Hepatology (Baltimore, Md *25*, 1022-1027.

Penna, A., Pilli, M., Zerbini, A., Orlandini, A., Mezzadri, S., Sacchelli, L., Missale, G., and Ferrari, C. (2007). Dysfunction and functional restoration of HCV-specific CD8 responses in chronic hepatitis C virus infection. Hepatology (Baltimore, Md *45*, 588-601.

Perrillo, R.P. (2001). Acute flares in chronic hepatitis B: the natural and unnatural history of an immunologically mediated liver disease. Gastroenterology *120*, 1009-1022.

Peterhans, E., Zanoni, R., and Bertoni, G. (1999). How to succeed as a virus: strategies for dealing with the immune system. Veterinary immunology and immunopathology *72*, 111-117.

Petrovas, C., Mueller, Y.M., Dimitriou, I.D., Bojczuk, P.M., Mounzer, K.C., Witek, J., Altman, J.D., and Katsikis, P.D. (2004). HIV-specific CD8+ T cells exhibit markedly reduced levels of Bcl-2 and Bcl-xL. J Immunol *172*, 4444-4453.

Petrovas, C., Mueller, Y.M., and Katsikis, P.D. (2005). Apoptosis of HIV-specific CD8+ T cells: an HIV evasion strategy. Cell death and differentiation *12 Suppl 1*, 859-870.

Pfeifer, J.D., Wick, M.J., Roberts, R.L., Findlay, K., Normark, S.J., and Harding, C.V. (1993). Phagocytic processing of bacterial antigens for class I MHC presentation to T cells. Nature *361*, 359-362.

Pichlmair, A., Schulz, O., Tan, C.P., Naslund, T.I., Liljestrom, P., Weber, F., and Reis e Sousa, C. (2006). RIG-I-mediated antiviral responses to single-stranded RNA bearing 5'-phosphates. Science (New York, NY *314*, 997-1001.

Piemonti, L., Monti, P., Allavena, P., Sironi, M., Soldini, L., Leone, B.E., Socci, C., and Di Carlo, V. (1999). Glucocorticoids affect human dendritic cell differentiation and maturation. J Immunol *162*, 6473-6481.

Pion, S., Christianson, G.J., Fontaine, P., Roopenian, D.C., and Perreault, C. (1999). Shaping the repertoire of cytotoxic T-lymphocyte responses: explanation for the

immunodominance effect whereby cytotoxic T lymphocytes specific for immunodominant antigens prevent recognition of nondominant antigens. Blood *93*, 952-962.

Platanias, L.C. (2005). Mechanisms of type-I- and type-II-interferon-mediated signalling. Nature reviews *5*, 375-386.

Plebanski, M., Flanagan, K.L., Lee, E.A., Reece, W.H., Hart, K., Gelder, C., Gillespie, G., Pinder, M., and Hill, A.V. (1999). Interleukin 10-mediated immunosuppression by a variant CD4 T cell epitope of Plasmodium falciparum. Immunity *10*, 651-660.

Ploegh, H.L. (1998). Viral strategies of immune evasion. Science (New York, NY *280*, 248-253.

Pohl-Koppe, A., Logigian, E.L., Steere, A.C., and Hafler, D.A. (1999). Cross-reactivity of Borrelia burgdorferi and myelin basic protein-specific T cells is not observed in borrelial encephalomyelitis. Cellular immunology *194*, 118-123.

Porritt, H.E., Gordon, K., and Petrie, H.T. (2003). Kinetics of steady-state differentiation and mapping of intrathymic-signaling environments by stem cell transplantation in nonirradiated mice. The Journal of experimental medicine *198*, 957-962.

Posnett, D.N., Engelhorn, M.E., and Houghton, A.N. (2005). Antiviral T cell responses: phalanx or multipronged attack? The Journal of experimental medicine *201*, 1881-1884.

Powell, J.D., Lerner, C.G., and Schwartz, R.H. (1999). Inhibition of cell cycle progression by rapamycin induces T cell clonal anergy even in the presence of costimulation. J Immunol *162*, 2775-2784.

Pozzi, L.A., Maciaszek, J.W., and Rock, K.L. (2005). Both dendritic cells and macrophages can stimulate naive CD8 T cells in vivo to proliferate, develop effector function, and differentiate into memory cells. J Immunol *175*, 2071-2081.

Prlic, M., Lefrancois, L., and Jameson, S.C. (2002). Multiple choices: regulation of memory CD8 T cell generation and homeostasis by interleukin (IL)-7 and IL-15. The Journal of experimental medicine *195*, F49-52.

Probst, H.C., Tschannen, K., Gallimore, A., Martinic, M., Basler, M., Dumrese, T., Jones, E., and van den Broek, M.F. (2003). Immunodominance of an antiviral cytotoxic T cell response is shaped by the kinetics of viral protein expression. J Immunol *171*, 5415-5422.

Provenzano, M., and Mocellin, S. (2007). Complementary techniques: validation of gene expression data by quantitative real time PCR. Advances in experimental medicine and biology *593*, 66-73.

Puthalakath, H., Huang, D.C., O'Reilly, L.A., King, S.M., and Strasser, A. (1999). The proapoptotic activity of the Bcl-2 family member Bim is regulated by interaction with the dynein motor complex. Molecular cell *3*, 287-296.

Puthalakath, H., Villunger, A., O'Reilly, L.A., Beaumont, J.G., Coultas, L., Cheney, R.E., Huang, D.C., and Strasser, A. (2001). Bmf: a proapoptotic BH3-only protein regulated by interaction with the myosin V actin motor complex, activated by anoikis. Science (New York, NY *293*, 1829-1832.

Radonic, A., Thulke, S., Mackay, I.M., Landt, O., Siegert, W., and Nitsche, A. (2004). Guideline to reference gene selection for quantitative real-time PCR. Biochemical and biophysical research communications *313*, 856-862.

Radziewicz, H., Ibegbu, C.C., Fernandez, M.L., Workowski, K.A., Obideen, K., Wehbi, M., Hanson, H.L., Steinberg, J.P., Masopust, D., Wherry, E.J., *et al.* (2007). Liver-infiltrating lymphocytes in chronic human hepatitis C virus infection display an exhausted phenotype with high levels of PD-1 and low levels of CD127 expression. Journal of virology *81*, 2545-2553.

Raghavan, A., Ogilvie, R.L., Reilly, C., Abelson, M.L., Raghavan, S., Vasdewani, J., Krathwohl, M., and Bohjanen, P.R. (2002). Genome-wide analysis of mRNA decay

in resting and activated primary human T lymphocytes. Nucleic acids research *30*, 5529-5538.

Raimondi, G., Zanoni, I., Citterio, S., Ricciardi-Castagnoli, P., and Granucci, F. (2006a). Induction of peripheral T cell tolerance by antigen-presenting B cells. I. Relevance of antigen presentation persistence. J Immunol *176*, 4012-4020.

Raimondi, G., Zanoni, I., Citterio, S., Ricciardi-Castagnoli, P., and Granucci, F. (2006b). Induction of peripheral T cell tolerance by antigen-presenting B cells. II. Chronic antigen presentation overrules antigen-presenting B cell activation. J Immunol *176*, 4021-4028.

Randolph, G.J. (2006). Migratory dendritic cells: sometimes simply ferries? Immunity *25*, 15-18.

Rasheed, A.U., Rahn, H.P., Sallusto, F., Lipp, M., and Muller, G. (2006). Follicular B helper T cell activity is confined to CXCR5(hi)ICOS(hi) CD4 T cells and is independent of CD57 expression. European journal of immunology *36*, 1892-1903.

Raue, H.P., Brien, J.D., Hammarlund, E., and Slifka, M.K. (2004). Activation of virus-specific CD8+ T cells by lipopolysaccharide-induced IL-12 and IL-18. J Immunol *173*, 6873-6881.

Rea, D., van Kooten, C., van Meijgaarden, K.E., Ottenhoff, T.H., Melief, C.J., and Offringa, R. (2000). Glucocorticoids transform CD40-triggering of dendritic cells into an alternative activation pathway resulting in antigen-presenting cells that secrete IL-10. Blood *95*, 3162-3167.

Redmond, W.L., Hernandez, J., and Sherman, L.A. (2003). Deletion of naive CD8 T cells requires persistent antigen and is not programmed by an initial signal from the tolerogenic APC. J Immunol *171*, 6349-6354.

Rehermann, B., Ferrari, C., Pasquinelli, C., and Chisari, F.V. (1996a). The hepatitis B virus persists for decades after patients' recovery from acute viral hepatitis despite

active maintenance of a cytotoxic T-lymphocyte response. Nature medicine *2*, 1104-1108.

Rehermann, B., Fowler, P., Sidney, J., Person, J., Redeker, A., Brown, M., Moss, B., Sette, A., and Chisari, F.V. (1995a). The cytotoxic T lymphocyte response to multiple hepatitis B virus polymerase epitopes during and after acute viral hepatitis. The Journal of experimental medicine *181*, 1047-1058.

Rehermann, B., Lau, D., Hoofnagle, J.H., and Chisari, F.V. (1996b). Cytotoxic T lymphocyte responsiveness after resolution of chronic hepatitis B virus infection. The Journal of clinical investigation *97*, 1655-1665.

Rehermann, B., Pasquinelli, C., Mosier, S.M., and Chisari, F.V. (1995b). Hepatitis B virus (HBV) sequence variation of cytotoxic T lymphocyte epitopes is not common in patients with chronic HBV infection. The Journal of clinical investigation *96*, 1527-1534.

Rehwinkel, J., Behm-Ansmant, I., Gatfield, D., and Izaurralde, E. (2005). A crucial role for GW182 and the DCP1:DCP2 decapping complex in miRNA-mediated gene silencing. RNA (New York, NY *11*, 1640-1647.

Reignat, S., Webster, G.J., Brown, D., Ogg, G.S., King, A., Seneviratne, S.L., Dusheiko, G., Williams, R., Maini, M.K., and Bertoletti, A. (2002). Escaping high viral load exhaustion: CD8 cells with altered tetramer binding in chronic hepatitis B virus infection. The Journal of experimental medicine *195*, 1089-1101.

Reiners, J.J., Jr., Caruso, J.A., Mathieu, P., Chelladurai, B., Yin, X.M., and Kessel, D. (2002). Release of cytochrome c and activation of pro-caspase-9 following lysosomal photodamage involves Bid cleavage. Cell death and differentiation *9*, 934-944.

Reinhardt, R.L., Kang, S.J., Liang, H.E., and Locksley, R.M. (2006). T helper cell effector fates--who, how and where? Current opinion in immunology *18*, 271-277.

Reinhardt, R.L., Khoruts, A., Merica, R., Zell, T., and Jenkins, M.K. (2001). Visualizing the generation of memory CD4 T cells in the whole body. Nature *410*, 101-105.

Restifo, N.P., Bacik, I., Irvine, K.R., Yewdell, J.W., McCabe, B.J., Anderson, R.W., Eisenlohr, L.C., Rosenberg, S.A., and Bennink, J.R. (1995). Antigen processing in vivo and the elicitation of primary CTL responses. J Immunol *154*, 4414-4422.

Ribeiro, R.M., Lo, A., and Perelson, A.S. (2002). Dynamics of hepatitis B virus infection. Microbes and infection / Institut Pasteur *4*, 829-835.

Riccardi, C., Bruscoli, S., and Migliorati, G. (2002). Molecular mechanisms of immunomodulatory activity of glucocorticoids. Pharmacol Res *45*, 361-368.

Ridge, J.P., Di Rosa, F., and Matzinger, P. (1998). A conditioned dendritic cell can be a temporal bridge between a CD4+ T-helper and a T-killer cell. Nature *393*, 474-478.

Riedl, P., Bertoletti, A., Lopes, R., Lemonnier, F., Reimann, J., and Schirmbeck, R. (2006). Distinct, cross-reactive epitope specificities of CD8 T cell responses are induced by natural hepatitis B surface antigen variants of different hepatitis B virus genotypes. J Immunol *176*, 4003-4011.

Rock, K.L. (1996). A new foreign policy: MHC class I molecules monitor the outside world. Immunology today *17*, 131-137.

Rock, K.L., Gamble, S., and Rothstein, L. (1990). Presentation of exogenous antigen with class I major histocompatibility complex molecules. Science (New York, NY *249*, 918-921.

Roelen, D.L., Schuurhuis, D.H., van den Boogaardt, D.E., Koekkoek, K., van Miert, P.P., van Schip, J.J., Laban, S., Rea, D., Melief, C.J., Offringa, R., *et al.* (2003). Prolongation of skin graft survival by modulation of the alloimmune response with alternatively activated dendritic cells. Transplantation *76*, 1608-1615.

Rogers, P.R., Song, J., Gramaglia, I., Killeen, N., and Croft, M. (2001). OX40 promotes Bcl-xL and Bcl-2 expression and is essential for long-term survival of CD4 T cells. Immunity *15*, 445-455.

Romani, N., Holzmann, S., Tripp, C.H., Koch, F., and Stoitzner, P. (2003). Langerhans cells - dendritic cells of the epidermis. Apmis *111*, 725-740.

Romisch, K. (1999). Surfing the Sec61 channel: bidirectional protein translocation across the ER membrane. Journal of cell science *112 (Pt 23)*, 4185-4191.

Rosler, C., Kock, J., Kann, M., Malim, M.H., Blum, H.E., Baumert, T.F., and von Weizsacker, F. (2005). APOBEC-mediated interference with hepadnavirus production. Hepatology (Baltimore, Md *42*, 301-309.

Rossner, M.T. (1992). Review: hepatitis B virus X-gene product: a promiscuous transcriptional activator. Journal of medical virology *36*, 101-117.

Rossol, S., Marinos, G., Carucci, P., Singer, M.V., Williams, R., and Naoumov, N.V. (1997). Interleukin-12 induction of Th1 cytokines is important for viral clearance in chronic hepatitis B. The Journal of clinical investigation *99*, 3025-3033.

Rotzschke, O., Falk, K., Stevanovic, S., Jung, G., and Rammensee, H.G. (1992). Peptide motifs of closely related HLA class I molecules encompass substantial differences. European journal of immunology *22*, 2453-2456.

Rowell, E.A., Walsh, M.C., and Wells, A.D. (2005). Opposing roles for the cyclin-dependent kinase inhibitor p27kip1 in the control of CD4+ T cell proliferation and effector function. J Immunol *174*, 3359-3368.

Roy-Proulx, G., Meunier, M.C., Lanteigne, A.M., Brochu, S., and Perreault, C. (2001). Immunodomination results from functional differences between competing CTL. European journal of immunology *31*, 2284-2292.

Rubio-Godoy, V., Dutoit, V., Rimoldi, D., Lienard, D., Lejeune, F., Speiser, D., Guillaume, P., Cerottini, J.C., Romero, P., and Valmori, D. (2001). Discrepancy between ELISPOT IFN-gamma secretion and binding of A2/peptide multimers to

TCR reveals interclonal dissociation of CTL effector function from TCR-peptide/MHC complexes half-life. Proceedings of the National Academy of Sciences of the United States of America *98*, 10302-10307.

Ruprecht, C.R., Gattorno, M., Ferlito, F., Gregorio, A., Martini, A., Lanzavecchia, A., and Sallusto, F. (2005). Coexpression of CD25 and CD27 identifies FoxP3+ regulatory T cells in inflamed synovia. The Journal of experimental medicine *201*, 1793-1803.

Sabbagh, L., Srokowski, C.C., Pulle, G., Snell, L.M., Sedgmen, B.J., Liu, Y., Tsitsikov, E.N., and Watts, T.H. (2006). A critical role for TNF receptor-associated factor 1 and Bim down-regulation in CD8 memory T cell survival. Proceedings of the National Academy of Sciences of the United States of America *103*, 18703-18708.

Sacre, K., Carcelain, G., Cassoux, N., Fillet, A.M., Costagliola, D., Vittecoq, D., Salmon, D., Amoura, Z., Katlama, C., and Autran, B. (2005). Repertoire, diversity, and differentiation of specific CD8 T cells are associated with immune protection against human cytomegalovirus disease. The Journal of experimental medicine *201*, 1999-2010.

Salio, M., Shepherd, D., Dunbar, P.R., Palmowski, M., Murphy, K., Wu, L., and Cerundolo, V. (2001). Mature dendritic cells prime functionally superior melan-A-specific CD8+ lymphocytes as compared with nonprofessional APC. J Immunol *167*, 1188-1197.

Sallusto, F., Geginat, J., and Lanzavecchia, A. (2004). Central memory and effector memory T cell subsets: function, generation, and maintenance. Annual review of immunology *22*, 745-763.

Sallusto, F., Lenig, D., Forster, R., Lipp, M., and Lanzavecchia, A. (1999). Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. Nature *401*, 708-712.

Samelson, L.E., Harford, J.B., and Klausner, R.D. (1985). Identification of the components of the murine T cell antigen receptor complex. Cell *43*, 223-231.

Samelson, L.E., Patel, M.D., Weissman, A.M., Harford, J.B., and Klausner, R.D. (1986). Antigen activation of murine T cells induces tyrosine phosphorylation of a polypeptide associated with the T cell antigen receptor. Cell *46*, 1083-1090.

Sandalova, E., Hislop, A.D., and Levitsky, V. (2006). T-cell receptor triggering differentially regulates bim expression in human lymphocytes from healthy individuals and patients with infectious mononucleosis. Human immunology *67*, 958-965.

Sandalova, E., Wei, C.H., Masucci, M.G., and Levitsky, V. (2004). Regulation of expression of Bcl-2 protein family member Bim by T cell receptor triggering. Proceedings of the National Academy of Sciences of the United States of America *101*, 3011-3016.

Santegoets, S.J., Schreurs, M.W., Masterson, A.J., Liu, Y.P., Goletz, S., Baumeister, H., Kueter, E.W., Lougheed, S.M., van den Eertwegh, A.J., Scheper, R.J., *et al.* (2006). In vitro priming of tumor-specific cytotoxic T lymphocytes using allogeneic dendritic cells derived from the human MUTZ-3 cell line. Cancer Immunol Immunother *55*, 1480-1490.

Sasai, M., Shingai, M., Funami, K., Yoneyama, M., Fujita, T., Matsumoto, M., and Seya, T. (2006). NAK-associated protein 1 participates in both the TLR3 and the cytoplasmic pathways in type I IFN induction. J Immunol *177*, 8676-8683.

Sauter, B., Albert, M.L., Francisco, L., Larsson, M., Somersan, S., and Bhardwaj, N. (2000). Consequences of cell death: exposure to necrotic tumor cells, but not primary tissue cells or apoptotic cells, induces the maturation of immunostimulatory dendritic cells. The Journal of experimental medicine *191*, 423-434.

Sawada, M., Hayes, P., and Matsuyama, S. (2003a). Cytoprotective membrane-permeable peptides designed from the Bax-binding domain of Ku70. Nature cell biology *5*, 352-357.

Sawada, M., Sun, W., Hayes, P., Leskov, K., Boothman, D.A., and Matsuyama, S. (2003b). Ku70 suppresses the apoptotic translocation of Bax to mitochondria. Nature cell biology *5*, 320-329.

Schoenberger, S.P. (2003). BLT for speed. Nature immunology *4*, 937-939.

Schoenberger, S.P., Toes, R.E., van der Voort, E.I., Offringa, R., and Melief, C.J. (1998). T-cell help for cytotoxic T lymphocytes is mediated by CD40-CD40L interactions. Nature *393*, 480-483.

Schroder, K., Hertzog, P.J., Ravasi, T., and Hume, D.A. (2004). Interferon-gamma: an overview of signals, mechanisms and functions. J Leukoc Biol *75*, 163-189.

Schwartz, R.H. (2003). T cell anergy. Annual review of immunology *21*, 305-334.

Sehra, S., and Dent, A.L. (2006). Caspase function and the immune system. Critical reviews in immunology *26*, 133-148.

Selin, L.K., Vergilis, K., Welsh, R.M., and Nahill, S.R. (1996). Reduction of otherwise remarkably stable virus-specific cytotoxic T lymphocyte memory by heterologous viral infections. The Journal of experimental medicine *183*, 2489-2499.

Selin, L.K., and Welsh, R.M. (2004). Plasticity of T cell memory responses to viruses. Immunity *20*, 5-16.

Sette, A., Vitiello, A., Reherman, B., Fowler, P., Nayersina, R., Kast, W.M., Melief, C.J., Oseroff, C., Yuan, L., Ruppert, J., *et al.* (1994). The relationship between class I binding affinity and immunogenicity of potential cytotoxic T cell epitopes. J Immunol *153*, 5586-5592.

Sharpe, A.H., and Freeman, G.J. (2002). The B7-CD28 superfamily. Nature reviews *2*, 116-126.

Shen, L., Sigal, L.J., Boes, M., and Rock, K.L. (2004). Important role of cathepsin S in generating peptides for TAP-independent MHC class I crosspresentation in vivo. Immunity *21*, 155-165.

Shortman, K., Egerton, M., Spangrude, G.J., and Scollay, R. (1990). The generation and fate of thymocytes. Seminars in immunology *2*, 3-12.

Shresta, S., Pham, C.T., Thomas, D.A., Graubert, T.A., and Ley, T.J. (1998). How do cytotoxic lymphocytes kill their targets? Current opinion in immunology *10*, 581-587.

Slifka, M.K., and Whitton, J.L. (2001). Functional avidity maturation of CD8(+) T cells without selection of higher affinity TCR. Nature immunology *2*, 711-717.

Smith, C.M., Belz, G.T., Wilson, N.S., Villadangos, J.A., Shortman, K., Carbone, F.R., and Heath, W.R. (2003). Cutting edge: conventional CD8 alpha+ dendritic cells are preferentially involved in CTL priming after footpad infection with herpes simplex virus-1. J Immunol *170*, 4437-4440.

Smith, C.M., Wilson, N.S., Waithman, J., Villadangos, J.A., Carbone, F.R., Heath, W.R., and Belz, G.T. (2004). Cognate CD4(+) T cell licensing of dendritic cells in CD8(+) T cell immunity. Nature immunology *5*, 1143-1148.

Song, Q., Kuang, Y., Dixit, V.M., and Vincenz, C. (1999). Boo, a novel negative regulator of cell death, interacts with Apaf-1. The EMBO journal *18*, 167-178.

Speiser, D.E., Lee, S.Y., Wong, B., Arron, J., Santana, A., Kong, Y.Y., Ohashi, P.S., and Choi, Y. (1997). A regulatory role for TRAF1 in antigen-induced apoptosis of T cells. The Journal of experimental medicine *185*, 1777-1783.

Spencer, J.V., and Braciale, T.J. (2000). Incomplete CD8(+) T lymphocyte differentiation as a mechanism for subdominant cytotoxic T lymphocyte responses to a viral antigen. The Journal of experimental medicine *191*, 1687-1698.

Spencer, J.V., Lockridge, K.M., Barry, P.A., Lin, G., Tsang, M., Penfold, M.E., and Schall, T.J. (2002). Potent immunosuppressive activities of cytomegalovirus-encoded interleukin-10. Journal of virology *76*, 1285-1292.

Sprengers, D., van der Molen, R.G., Kusters, J.G., Kwekkeboom, J., van der Laan, L.J., Niesters, H.G., Kuipers, E.J., De Man, R.A., Schalm, S.W., and Janssen, H.L. (2005). Flow cytometry of fine-needle-aspiration biopsies: a new method to monitor

the intrahepatic immunological environment in chronic viral hepatitis. Journal of viral hepatitis *12*, 507-512.

Sprent, J., and Tough, D.F. (2001). T cell death and memory. Science (New York, NY *293*, 245-248.

Stark, G.R., Kerr, I.M., Williams, B.R., Silverman, R.H., and Schreiber, R.D. (1998). How cells respond to interferons. Annual review of biochemistry *67*, 227-264.

Steinman, R.M., Granelli-Piperno, A., Pope, M., Trumpfheller, C., Ignatius, R., Arrode, G., Racz, P., and Tenner-Racz, K. (2003a). The interaction of immunodeficiency viruses with dendritic cells. Current topics in microbiology and immunology *276*, 1-30.

Steinman, R.M., Hawiger, D., and Nussenzweig, M.C. (2003b). Tolerogenic dendritic cells. Annual review of immunology *21*, 685-711.

Steinman, R.M., Pack, M., and Inaba, K. (1997). Dendritic cells in the T-cell areas of lymphoid organs. Immunological reviews *156*, 25-37.

Stephan, W., Prince, A.M., and Brotman, B. (1984). Modulation of hepatitis B infection by intravenous application of an immunoglobulin preparation that contains antibodies to hepatitis B e and core antigens but not to hepatitis B surface antigen. Journal of virology *51*, 420-424.

Stoka, V., Turk, B., Schendel, S.L., Kim, T.H., Cirman, T., Snipas, S.J., Ellerby, L.M., Bredesen, D., Freeze, H., Abrahamson, M., *et al.* (2001). Lysosomal protease pathways to apoptosis. Cleavage of bid, not pro-caspases, is the most likely route. The Journal of biological chemistry *276*, 3149-3157.

Stoop, J.N., van der Molen, R.G., Baan, C.C., van der Laan, L.J., Kuipers, E.J., Kusters, J.G., and Janssen, H.L. (2005). Regulatory T cells contribute to the impaired immune response in patients with chronic hepatitis B virus infection. Hepatology (Baltimore, Md *41*, 771-778.

Strasser, A., Harris, A.W., Huang, D.C., Krammer, P.H., and Cory, S. (1995). Bcl-2 and Fas/APO-1 regulate distinct pathways to lymphocyte apoptosis. The EMBO journal *14*, 6136-6147.

Su, M.A., and Anderson, M.S. (2004). Aire: an update. Current opinion in immunology *16*, 746-752.

Tartaglia, L.A., Goeddel, D.V., Reynolds, C., Figari, I.S., Weber, R.F., Fendly, B.M., and Palladino, M.A., Jr. (1993). Stimulation of human T-cell proliferation by specific activation of the 75-kDa tumor necrosis factor receptor. J Immunol *151*, 4637-4641.

Tassopoulos, N.C., Papaevangelou, G.J., Sjogren, M.H., Roumeliotou-Karayannis, A., Gerin, J.L., and Purcell, R.H. (1987). Natural history of acute hepatitis B surface antigen-positive hepatitis in Greek adults. Gastroenterology *92*, 1844-1850.

Tavakoli, S., Schwerin, W., Rohwer, A., Hoffmann, S., Weyer, S., Weth, R., Meisel, H., Diepolder, H., Geissler, M., Galle, P.R., *et al.* (2004). Phenotype and function of monocyte derived dendritic cells in chronic hepatitis B virus infection. The Journal of general virology *85*, 2829-2836.

Teague, T.K., Hildeman, D., Kedl, R.M., Mitchell, T., Rees, W., Schaefer, B.C., Bender, J., Kappler, J., and Marrack, P. (1999). Activation changes the spectrum but not the diversity of genes expressed by T cells. Proceedings of the National Academy of Sciences of the United States of America *96*, 12691-12696.

Tewari, K., Sacha, J., Gao, X., and Suresh, M. (2004). Effect of chronic viral infection on epitope selection, cytokine production, and surface phenotype of CD8 T cells and the role of IFN-gamma receptor in immune regulation. J Immunol *172*, 1491-1500.

Thellin, O., Zorzi, W., Lakaye, B., De Borman, B., Coumans, B., Hennen, G., Grisar, T., Igout, A., and Heinen, E. (1999). Housekeeping genes as internal standards: use and limits. Journal of biotechnology *75*, 291-295.

Theofilopoulos, A.N., Baccala, R., Beutler, B., and Kono, D.H. (2005). Type I interferons (alpha/beta) in immunity and autoimmunity. Annual review of immunology *23*, 307-336.

Thimme, R., Wieland, S., Steiger, C., Ghrayeb, J., Reimann, K.A., Purcell, R.H., and Chisari, F.V. (2003). CD8(+) T cells mediate viral clearance and disease pathogenesis during acute hepatitis B virus infection. Journal of virology *77*, 68-76.

Thome, M., Schneider, P., Hofmann, K., Fickenscher, H., Meinl, E., Neipel, F., Mattmann, C., Burns, K., Bodmer, J.L., Schroter, M., *et al.* (1997). Viral FLICE-inhibitory proteins (FLIPs) prevent apoptosis induced by death receptors. Nature *386*, 517-521.

Thornberry, N.A., and Lazebnik, Y. (1998). Caspases: enemies within. Science (New York, NY *281*, 1312-1316.

Tobery, T.W., Wang, S., Wang, X.M., Neeper, M.P., Jansen, K.U., McClements, W.L., and Caulfield, M.J. (2001). A simple and efficient method for the monitoring of antigen-specific T cell responses using peptide pool arrays in a modified ELISpot assay. Journal of immunological methods *254*, 59-66.

Tortorella, D., Gewurz, B.E., Furman, M.H., Schust, D.J., and Ploegh, H.L. (2000). Viral subversion of the immune system. Annual review of immunology *18*, 861-926.

Trop, S., De Sepulveda, P., Zuniga-Pflucker, J.C., and Rottapel, R. (2001). Overexpression of suppressor of cytokine signaling-1 impairs pre-T-cell receptor-induced proliferation but not differentiation of immature thymocytes. Blood *97*, 2269-2277.

Truant, R., Antunovic, J., Greenblatt, J., Prives, C., and Cromlish, J.A. (1995). Direct interaction of the hepatitis B virus HBx protein with p53 leads to inhibition by HBx of p53 response element-directed transactivation. Journal of virology *69*, 1851-1859.

Tsai, S.L., Chen, P.J., Lai, M.Y., Yang, P.M., Sung, J.L., Huang, J.H., Hwang, L.H., Chang, T.H., and Chen, D.S. (1992). Acute exacerbations of chronic type B hepatitis

are accompanied by increased T cell responses to hepatitis B core and e antigens. Implications for hepatitis B e antigen seroconversion. The Journal of clinical investigation *89*, 87-96.

Tscharke, D.C., Karupiah, G., Zhou, J., Palmore, T., Irvine, K.R., Haeryfar, S.M., Williams, S., Sidney, J., Sette, A., Bennink, J.R., *et al.* (2005). Identification of poxvirus CD8+ T cell determinants to enable rational design and characterization of smallpox vaccines. The Journal of experimental medicine *201*, 95-104.

Tsomides, T.J., Aldovini, A., Johnson, R.P., Walker, B.D., Young, R.A., and Eisen, H.N. (1994). Naturally processed viral peptides recognized by cytotoxic T lymphocytes on cells chronically infected by human immunodeficiency virus type 1. The Journal of experimental medicine *180*, 1283-1293.

Tsujimoto, Y., Cossman, J., Jaffe, E., and Croce, C.M. (1985). Involvement of the bcl-2 gene in human follicular lymphoma. Science (New York, NY *228*, 1440-1443.

Tsurumi, T., Fujita, M., and Kudoh, A. (2005). Latent and lytic Epstein-Barr virus replication strategies. Reviews in medical virology *15*, 3-15.

Tusher, V.G., Tibshirani, R., and Chu, G. (2001). Significance analysis of microarrays applied to the ionizing radiation response. Proceedings of the National Academy of Sciences of the United States of America *98*, 5116-5121.

U, M., Miyashita, T., Shikama, Y., Tadokoro, K., and Yamada, M. (2001). Molecular cloning and characterization of six novel isoforms of human Bim, a member of the proapoptotic Bcl-2 family. FEBS letters *509*, 135-141.

Umene, K., and Sakaoka, H. (1999). Evolution of herpes simplex virus type 1 under herpesviral evolutionary processes. Archives of virology *144*, 637-656.

Unanue, E.R. (2007). Ito cells, stellate cells, and myofibroblasts: new actors in antigen presentation. Immunity *26*, 9-10.

Untergasser, A., Zedler, U., Langenkamp, A., Hosel, M., Quasdorff, M., Esser, K., Dienes, H.P., Tappertzhofen, B., Kolanus, W., and Protzer, U. (2006). Dendritic cells

take up viral antigens but do not support the early steps of hepatitis B virus infection. Hepatology (Baltimore, Md *43*, 539-547.

Urbani, S., Amadei, B., Fisicaro, P., Pilli, M., Missale, G., Bertoletti, A., and Ferrari, C. (2005). Heterologous T cell immunity in severe hepatitis C virus infection. The Journal of experimental medicine *201*, 675-680.

Uren, R.T., Dewson, G., Chen, L., Coyne, S.C., Huang, D.C., Adams, J.M., and Kluck, R.M. (2007). Mitochondrial permeabilization relies on BH3 ligands engaging multiple prosurvival Bcl-2 relatives, not Bak. The Journal of cell biology *177*, 277-287.

Utz, U., Banks, D., Jacobson, S., and Biddison, W.E. (1996). Analysis of the T-cell receptor repertoire of human T-cell leukemia virus type 1 (HTLV-1) Tax-specific CD8+ cytotoxic T lymphocytes from patients with HTLV-1-associated disease: evidence for oligoclonal expansion. Journal of virology *70*, 843-851.

Valdez, Y., Mah, W., Winslow, M.M., Xu, L., Ling, P., and Townsend, S.E. (2002). Major histocompatibility complex class II presentation of cell-associated antigen is mediated by CD8alpha+ dendritic cells in vivo. The Journal of experimental medicine *195*, 683-694.

van der Molen, R.G., Sprengers, D., Binda, R.S., de Jong, E.C., Niesters, H.G., Kusters, J.G., Kwekkeboom, J., and Janssen, H.L. (2004). Functional impairment of myeloid and plasmacytoid dendritic cells of patients with chronic hepatitis B. Hepatology (Baltimore, Md *40*, 738-746.

van der Most, R.G., Murali-Krishna, K., Whitton, J.L., Oseroff, C., Alexander, J., Southwood, S., Sidney, J., Chesnut, R.W., Sette, A., and Ahmed, R. (1998). Identification of Db- and Kb-restricted subdominant cytotoxic T-cell responses in lymphocytic choriomeningitis virus-infected mice. Virology *240*, 158-167.

van der Most, R.G., Sette, A., Oseroff, C., Alexander, J., Murali-Krishna, K., Lau, L.L., Southwood, S., Sidney, J., Chesnut, R.W., Matloubian, M., *et al.* (1996). Analysis of cytotoxic T cell responses to dominant and subdominant epitopes during

acute and chronic lymphocytic choriomeningitis virus infection. J Immunol *157*, 5543-5554.

van Endert, P., and Villadangos, J.A. (2007). Antigen processing and recognition. Current opinion in immunology *19*, 63-65.

Van Gelder, R.N., von Zastrow, M.E., Yool, A., Dement, W.C., Barchas, J.D., and Eberwine, J.H. (1990). Amplified RNA synthesized from limited quantities of heterogeneous cDNA. Proceedings of the National Academy of Sciences of the United States of America *87*, 1663-1667.

van Leeuwen, E.M., de Bree, G.J., Remmerswaal, E.B., Yong, S.L., Tesselaar, K., ten Berge, I.J., and van Lier, R.A. (2005). IL-7 receptor alpha chain expression distinguishes functional subsets of virus-specific human CD8+ T cells. Blood *106*, 2091-2098.

van Mierlo, G.J., den Boer, A.T., Medema, J.P., van der Voort, E.I., Fransen, M.F., Offringa, R., Melief, C.J., and Toes, R.E. (2002). CD40 stimulation leads to effective therapy of CD40(-) tumors through induction of strong systemic cytotoxic T lymphocyte immunity. Proceedings of the National Academy of Sciences of the United States of America *99*, 5561-5566.

van Stipdonk, M.J., Lemmens, E.E., and Schoenberger, S.P. (2001). Naive CTLs require a single brief period of antigenic stimulation for clonal expansion and differentiation. Nature immunology *2*, 423-429.

Varadhachary, A.S., and Salgame, P. (1998). CD95 mediated T cell apoptosis and its relevance to immune deviation. Oncogene *17*, 3271-3276.

Verthelyi, D., Wang, V.W., Lifson, J.D., and Klinman, D.M. (2004). CpG oligodeoxynucleotides improve the response to hepatitis B immunization in healthy and SIV-infected rhesus macaques. AIDS (London, England) *18*, 1003-1008.

Vezys, V., Masopust, D., Kemball, C.C., Barber, D.L., O'Mara, L.A., Larsen, C.P., Pearson, T.C., Ahmed, R., and Lukacher, A.E. (2006). Continuous recruitment of

naive T cells contributes to heterogeneity of antiviral CD8 T cells during persistent infection. The Journal of experimental medicine *203*, 2263-2269.

Vilasco, M., Larrea, E., Vitour, D., Dabo, S., Breiman, A., Regnault, B., Riezu, J.I., Eid, P., Prieto, J., and Meurs, E.F. (2006). The protein kinase IKKepsilon can inhibit HCV expression independently of IFN and its own expression is downregulated in HCV-infected livers. Hepatology (Baltimore, Md *44*, 1635-1647.

Villadangos, J.A. (2007). Hold on, the monocytes are coming! Immunity *26*, 390-392.

Villadangos, J.A., and Heath, W.R. (2005). Life cycle, migration and antigen presenting functions of spleen and lymph node dendritic cells: limitations of the Langerhans cells paradigm. Seminars in immunology *17*, 262-272.

Villadangos, J.A., Heath, W.R., and Carbone, F.R. (2007). Outside looking in: the inner workings of the cross-presentation pathway within dendritic cells. Trends in immunology *28*, 45-47.

Villarreal, L.P., Defilippis, V.R., and Gottlieb, K.A. (2000). Acute and persistent viral life strategies and their relationship to emerging diseases. Virology *272*, 1-6.

Vine, A.M., Heaps, A.G., Kaftantzi, L., Mosley, A., Asquith, B., Witkover, A., Thompson, G., Saito, M., Goon, P.K., Carr, L., *et al.* (2004). The role of CTLs in persistent viral infection: cytolytic gene expression in CD8+ lymphocytes distinguishes between individuals with a high or low proviral load of human T cell lymphotropic virus type 1. J Immunol *173*, 5121-5129.

von Boehmer, H., Aifantis, I., Gounari, F., Azogui, O., Haughn, L., Apostolou, I., Jaeckel, E., Grassi, F., and Klein, L. (2003). Thymic selection revisited: how essential is it? Immunological reviews *191*, 62-78.

Vossen, M.T., Westerhout, E.M., Soderberg-Naucler, C., and Wiertz, E.J. (2002). Viral immune evasion: a masterpiece of evolution. Immunogenetics *54*, 527-542.

Vremec, D., Pooley, J., Hochrein, H., Wu, L., and Shortman, K. (2000). CD4 and CD8 expression by dendritic cell subtypes in mouse thymus and spleen. J Immunol *164*, 2978-2986.

Vremec, D., Zorbas, M., Scollay, R., Saunders, D.J., Ardavin, C.F., Wu, L., and Shortman, K. (1992). The surface phenotype of dendritic cells purified from mouse thymus and spleen: investigation of the CD8 expression by a subpopulation of dendritic cells. The Journal of experimental medicine *176*, 47-58.

Wallach, D., Kovalenko, A.V., Varfolomeev, E.E., and Boldin, M.P. (1998). Death-inducing functions of ligands of the tumor necrosis factor family: a Sanhedrin verdict. Current opinion in immunology *10*, 279-288.

Wang, E., Miller, L.D., Ohnmacht, G.A., Liu, E.T., and Marincola, F.M. (2000). High-fidelity mRNA amplification for gene profiling. Nature biotechnology *18*, 457-459.

Wang, J.P., Asher, D.R., Chan, M., Kurt-Jones, E.A., and Finberg, R.W. (2007). Cutting Edge: Antibody-mediated TLR7-dependent recognition of viral RNA. J Immunol *178*, 3363-3367.

Wang, K., Yin, X.M., Chao, D.T., Milliman, C.L., and Korsmeyer, S.J. (1996). BID: a novel BH3 domain-only death agonist. Genes & development *10*, 2859-2869.

Wang, X.W., Forrester, K., Yeh, H., Feitelson, M.A., Gu, J.R., and Harris, C.C. (1994). Hepatitis B virus X protein inhibits p53 sequence-specific DNA binding, transcriptional activity, and association with transcription factor ERCC3. Proceedings of the National Academy of Sciences of the United States of America *91*, 2230-2234.

Wange, R.L., Kong, A.N., and Samelson, L.E. (1992). A tyrosine-phosphorylated 70-kDa protein binds a photoaffinity analogue of ATP and associates with both the zeta chain and CD3 components of the activated T cell antigen receptor. The Journal of biological chemistry *267*, 11685-11688.

Ware, C.F. (2005). Network communications: lymphotoxins, LIGHT, and TNF. Annual review of immunology *23*, 787-819.

Wathelet, M.G., Lin, C.H., Parekh, B.S., Ronco, L.V., Howley, P.M., and Maniatis, T. (1998). Virus infection induces the assembly of coordinately activated transcription factors on the IFN-beta enhancer in vivo. Molecular cell *1*, 507-518.

Weber, A., Paschen, S.A., Heger, K., Wilfling, F., Frankenberg, T., Bauerschmitt, H., Seiffert, B.M., Kirschnek, S., Wagner, H., and Hacker, G. (2007). BimS-induced apoptosis requires mitochondrial localization but not interaction with anti-apoptotic Bcl-2 proteins. The Journal of cell biology *177*, 625-636.

Webster, G.J., Reignat, S., Brown, D., Ogg, G.S., Jones, L., Seneviratne, S.L., Williams, R., Dusheiko, G., and Bertoletti, A. (2004). Longitudinal analysis of CD8+ T cells specific for structural and nonstructural hepatitis B virus proteins in patients with chronic hepatitis B: implications for immunotherapy. Journal of virology *78*, 5707-5719.

Webster, G.J., Reignat, S., Maini, M.K., Whalley, S.A., Ogg, G.S., King, A., Brown, D., Amlot, P.L., Williams, R., Vergani, D., *et al.* (2000). Incubation phase of acute hepatitis B in man: dynamic of cellular immune mechanisms. Hepatology (Baltimore, Md *32*, 1117-1124.

Weinberger, K.M., Wiedenmann, E., Bohm, S., and Jilg, W. (2000). Sensitive and accurate quantitation of hepatitis B virus DNA using a kinetic fluorescence detection system (TaqMan PCR). Journal of virological methods *85*, 75-82.

Weissman, A.M., Samelson, L.E., and Klausner, R.D. (1986). A new subunit of the human T-cell antigen receptor complex. Nature *324*, 480-482.

Weissmann, C., and Weber, H. (1986). The interferon genes. Progress in nucleic acid research and molecular biology *33*, 251-300.

Welsh, R.M., and Selin, L.K. (2002). No one is naive: the significance of heterologous T-cell immunity. Nature reviews *2*, 417-426.

Weninger, W., Manjunath, N., and von Andrian, U.H. (2002). Migration and differentiation of CD8+ T cells. Immunological reviews *186*, 221-233.

Wherry, E.J., and Ahmed, R. (2004). Memory CD8 T-cell differentiation during viral infection. Journal of virology *78*, 5535-5545.

Wherry, E.J., Blattman, J.N., Murali-Krishna, K., van der Most, R., and Ahmed, R. (2003). Viral persistence alters CD8 T-cell immunodominance and tissue distribution and results in distinct stages of functional impairment. Journal of virology *77*, 4911-4927.

Wieland, S.F., Guidotti, L.G., and Chisari, F.V. (2000). Intrahepatic induction of alpha/beta interferon eliminates viral RNA-containing capsids in hepatitis B virus transgenic mice. Journal of virology *74*, 4165-4173.

Wiertz, E.J., Jones, T.R., Sun, L., Bogyo, M., Geuze, H.J., and Ploegh, H.L. (1996a). The human cytomegalovirus US11 gene product dislocates MHC class I heavy chains from the endoplasmic reticulum to the cytosol. Cell *84*, 769-779.

Wiertz, E.J., Tortorella, D., Bogyo, M., Yu, J., Mothes, W., Jones, T.R., Rapoport, T.A., and Ploegh, H.L. (1996b). Sec61-mediated transfer of a membrane protein from the endoplasmic reticulum to the proteasome for destruction. Nature *384*, 432-438.

Will, H., Reiser, W., Weimer, T., Pfaff, E., Buscher, M., Sprengel, R., Cattaneo, R., and Schaller, H. (1987). Replication strategy of human hepatitis B virus. Journal of virology *61*, 904-911.

Williams, M.A., Tyznik, A.J., and Bevan, M.J. (2006). Interleukin-2 signals during priming are required for secondary expansion of CD8+ memory T cells. Nature *441*, 890-893.

Willis, S.N., Fletcher, J.I., Kaufmann, T., van Delft, M.F., Chen, L., Czabotar, P.E., Ierino, H., Lee, E.F., Fairlie, W.D., Bouillet, P., *et al.* (2007). Apoptosis initiated when BH3 ligands engage multiple Bcl-2 homologs, not Bax or Bak. Science (New York, NY *315*, 856-859.

Wills, M.R., Carmichael, A.J., Mynard, K., Jin, X., Weekes, M.P., Plachter, B., and Sissons, J.G. (1996). The human cytotoxic T-lymphocyte (CTL) response to cytomegalovirus is dominated by structural protein pp65: frequency, specificity, and T-cell receptor usage of pp65-specific CTL. Journal of virology *70*, 7569-7579.

Winau, F., Hegasy, G., Weiskirchen, R., Weber, S., Cassan, C., Sieling, P.A., Modlin, R.L., Liblau, R.S., Gressner, A.M., and Kaufmann, S.H. (2007). Ito cells are liver-resident antigen-presenting cells for activating T cell responses. Immunity *26*, 117-129.

Wojciechowski, S., Jordan, M.B., Zhu, Y., White, J., Zajac, A.J., and Hildeman, D.A. (2006). Bim mediates apoptosis of CD127(lo) effector T cells and limits T cell memory. European journal of immunology *36*, 1694-1706.

Woltman, A.M., de Fijter, J.W., Kamerling, S.W., Paul, L.C., Daha, M.R., and van Kooten, C. (2000). The effect of calcineurin inhibitors and corticosteroids on the differentiation of human dendritic cells. European journal of immunology *30*, 1807-1812.

Wong, P., and Pamer, E.G. (2001). Cutting edge: antigen-independent CD8 T cell proliferation. J Immunol *166*, 5864-5868.

Xia, C.Q., Peng, R., Beato, F., and Clare-Salzler, M.J. (2005). Dexamethasone induces IL-10-producing monocyte-derived dendritic cells with durable immaturity. Scandinavian journal of immunology *62*, 45-54.

Xiong, Y., Luscher, M.A., Altman, J.D., Hulsey, M., Robinson, H.L., Ostrowski, M., Barber, B.H., and MacDonald, K.S. (2001). Simian immunodeficiency virus (SIV) infection of a rhesus macaque induces SIV-specific CD8(+) T cells with a defect in effector function that is reversible on extended interleukin-2 incubation. Journal of virology *75*, 3028-3033.

Xu, D., Fu, J., Jin, L., Zhang, H., Zhou, C., Zou, Z., Zhao, J.M., Zhang, B., Shi, M., Ding, X., *et al.* (2006). Circulating and liver resident CD4+CD25+ regulatory T cells

actively influence the antiviral immune response and disease progression in patients with hepatitis B. J Immunol *177*, 739-747.

Xu, X.N., Screaton, G.R., Gotch, F.M., Dong, T., Tan, R., Almond, N., Walker, B., Stebbings, R., Kent, K., Nagata, S., *et al.* (1997). Evasion of cytotoxic T lymphocyte (CTL) responses by nef-dependent induction of Fas ligand (CD95L) expression on simian immunodeficiency virus-infected cells. The Journal of experimental medicine *186*, 7-16.

Xu, X.N., Screaton, G.R., and McMichael, A.J. (2001). Virus infections: escape, resistance, and counterattack. Immunity *15*, 867-870.

Yang, E., Zha, J., Jockel, J., Boise, L.H., Thompson, C.B., and Korsmeyer, S.J. (1995). Bad, a heterodimeric partner for Bcl-XL and Bcl-2, displaces Bax and promotes cell death. Cell *80*, 285-291.

Yang, Y.H., Dudoit, S., Luu, P., Lin, D.M., Peng, V., Ngai, J., and Speed, T.P. (2002). Normalization for cDNA microarray data: a robust composite method addressing single and multiple slide systematic variation. Nucleic acids research *30*, e15.

Ye, P., and Kirschner, D.E. (2002). Measuring emigration of human thymocytes by T-cell receptor excision circles. Critical reviews in immunology *22*, 483-497.

Yeh, W.C., Itie, A., Elia, A.J., Ng, M., Shu, H.B., Wakeham, A., Mirtsos, C., Suzuki, N., Bonnard, M., Goeddel, D.V., *et al.* (2000). Requirement for Casper (c-FLIP) in regulation of death receptor-induced apoptosis and embryonic development. Immunity *12*, 633-642.

Yewdell, J.W. (2006). Confronting complexity: real-world immunodominance in antiviral CD8+ T cell responses. Immunity *25*, 533-543.

Yewdell, J.W., and Bennink, J.R. (1992). Cell biology of antigen processing and presentation to major histocompatibility complex class I molecule-restricted T lymphocytes. Advances in immunology *52*, 1-123.

Yoneyama, H., Matsuno, K., Toda, E., Nishiwaki, T., Matsuo, N., Nakano, A., Narumi, S., Lu, B., Gerard, C., Ishikawa, S., *et al.* (2005). Plasmacytoid DCs help lymph node DCs to induce anti-HSV CTLs. The Journal of experimental medicine *202*, 425-435.

Yoneyama, M., Kikuchi, M., Natsukawa, T., Shinobu, N., Imaizumi, T., Miyagishi, M., Taira, K., Akira, S., and Fujita, T. (2004). The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. Nature immunology *5*, 730-737.

York, I.A., Roop, C., Andrews, D.W., Riddell, S.R., Graham, F.L., and Johnson, D.C. (1994). A cytosolic herpes simplex virus protein inhibits antigen presentation to CD8+ T lymphocytes. Cell *77*, 525-535.

You, H., Pellegrini, M., Tsuchihara, K., Yamamoto, K., Hacker, G., Erlacher, M., Villunger, A., and Mak, T.W. (2006). FOXO3a-dependent regulation of Puma in response to cytokine/growth factor withdrawal. The Journal of experimental medicine *203*, 1657-1663.

Yu, J., Chen, H., Horton, H., Bansal, A., McElrath, J.M., Reichman, R., Goepfert, P., and Jin, X. (2006). Interleukin-2 reconstitutes defective human immunodeficiency virus (HIV), and cytomegalovirus (CMV) specific CD8+ T cell proliferation in HIV infection. Journal of medical virology *78*, 1147-1157.

Zajac, A.J., Blattman, J.N., Murali-Krishna, K., Sourdive, D.J., Suresh, M., Altman, J.D., and Ahmed, R. (1998). Viral immune evasion due to persistence of activated T cells without effector function. The Journal of experimental medicine *188*, 2205-2213.

Zerbini, L.F., and Libermann, T.A. (2005). Life and death in cancer. GADD45 alpha and gamma are critical regulators of NF-kappaB mediated escape from programmed cell death. Cell cycle (Georgetown, Tex *4*, 18-20.

Zha, J., Harada, H., Yang, E., Jockel, J., and Korsmeyer, S.J. (1996). Serine phosphorylation of death agonist BAD in response to survival factor results in binding to 14-3-3 not BCL-X(L). Cell *87*, 619-628.

Zhang, X., Chen, Z., Huang, H., Gordon, J.R., and Xiang, J. (2002). DNA microarray analysis of the gene expression profiles of naive versus activated tumor-specific T cells. Life sciences *71*, 3005-3017.

Zhao, X., Deak, E., Soderberg, K., Linehan, M., Spezzano, D., Zhu, J., Knipe, D.M., and Iwasaki, A. (2003). Vaginal submucosal dendritic cells, but not Langerhans cells, induce protective Th1 responses to herpes simplex virus-2. The Journal of experimental medicine *197*, 153-162.

Zhu, Y., Swanson, B.J., Wang, M., Hildeman, D.A., Schaefer, B.C., Liu, X., Suzuki, H., Mihara, K., Kappler, J., and Marrack, P. (2004). Constitutive association of the proapoptotic protein Bim with Bcl-2-related proteins on mitochondria in T cells. Proceedings of the National Academy of Sciences of the United States of America *101*, 7681-7686.

Zong, W.X., Lindsten, T., Ross, A.J., MacGregor, G.R., and Thompson, C.B. (2001). BH3-only proteins that bind pro-survival Bcl-2 family members fail to induce apoptosis in the absence of Bax and Bak. Genes & development *15*, 1481-1486.

Zoulim, F., and Seeger, C. (1994). Reverse transcription in hepatitis B viruses is primed by a tyrosine residue of the polymerase. Journal of virology *68*, 6-13.

The Journal of Immunology

Greater CD8$^+$ TCR Heterogeneity and Functional Flexibility in HIV-2 Compared to HIV-1 Infection[1]

A. Ross Lopes,*† Assan Jaye,‡ Lucy Dorrell,‡§ Sehu Sabally,‡ Abraham Alabi,‡ Nicola A. Jones,¶ Darren R. Flower,¶ Anne De Groot,‖ Phillipa Newton,† R. Monica Lascar,† Ian Williams,† Hilton Whittle,‡ Antonio Bertoletti,* Persephone Borrow,¶ and Mala K. Maini[2]*†

0022-1767/03/$02.00